The Immunological Role of Enterocytes and Probiotics in Necrotizing Enterocolitis

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This thesis is submitted for the degree of Master of Science

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May 2014

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Abstract

Necrotizing enterocolitis (NEC) is an important and sometimes fatal intestinal disease that primarily affects premature neonates and although it has been extensively investigated, the complete pathogenesis of NEC remains unclear. Important immunological factors such as interleukin 8 (IL-8), toll like receptor 4 (TLR4), cluster of differentiation 14 (CD14), lipopolysaccharide (LPS), and also bacteria from the *Enterobacteriaceae* family have been implicated in the pathogenesis of NEC while probiotics have been used clinically as a prophylactic treatment. In order to produce more effective treatments, it is important to understand the pathophysiology of NEC and also the mechanism of action of probiotics. We demonstrate here that human colonic epithelial cells (HT-29 cells) are able to produce significantly increased amounts of IL-8 in response to purified bacterial LPS and 12 strains of *Enterobacteriaceae*, isolated from clinical NEC specimens, compared to unstimulated controls. This response was time and dose-dependent, and was confirmed by the use of another colonic epithelial cell line (Caco-2 cells). Two strains of clinically utilised probiotic bacteria (*Bifidobacterium infantis* and *Lactobacillus acidophilus*) did not induce IL-8 production by either intestinal epithelial cell (IEC). The addition of live *B. infantis* or a Gram positive cell wall component, lipoteichoic acid (LTA), was able to significantly reduce the IL-8 response seen during an LPS stimulation of HT-29 cells. No reduction in IL-8 production was demonstrated by heat inactivated probiotics or live *L. acidophilus*. CD14 and TLR4 were expressed by HT-29 cells and it was found that LPS induced IL-8 production was CD14-dependent. It has previously been shown that LPS and bacteria can induce an IL-8 response from intestinal epithelial cells and that probiotic bacteria are effective at reducing both this response and also the incidence of NEC. However, this is the first report of an immunological mechanism using these NEC-associated *Enterobacteriaceae* and probiotic strains and intestinal epithelial cells.

Acknowledgements

First off, I would like to thank my supervisors, Dr. Michelle McConnell and Dr. Roslyn Kemp, for the amazing opportunity that they have given me, for all the guidance I have received and all the patience that they have had with me. Secondly, I would like to thank Dr. Heather Brooks for the input she has had, and the expertise that she has shared. I would like to give a tremendous thank you to Clare Fitzpatrick and Sonya Mros for all the help that they have given me and the time and effort that they have put in. I would like to extend my thanks to Dr. Jo Kirman, Michelle Wilson, Vivienne Young, Colin Davies, Emily Waugh, Liam Brennan, Edward Taylor, Andy Highton, Elliott Dunn, Georgia Moana Bell, Clare Burn and also Pia Steigler for all the support and help that they have given me. I would like to thank all of my fellow students for being such an outstanding group of friends and supporting me during my journey. Also, I would like to thank the whole of the department of Microbiology and Immunology, Otago. It is truly a great place to work and study, with equally great people. Last but certainly not least, I would also like to say thank you to my flatmates, my friends and also my family especially my Mother, Diane Joyce and my partner, Jacqueline Legros for all the love and support that they have given me along the way.

Cheers.

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List of abbreviations

%	Percent
®	Registered and authorised company logo
°C	Degrees Celsius
µg	Micrograms
µL	Microlitres
16S rRNA	Ribosomal ribonucleic acid from the 16S subunit
2-ME	2-Mercaptoethanol
ANOVA	Analysis of variance
API	Analytical profile index
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CARD15	Caspase activation and recruitment domain 15
CD14	Cluster of differentiation 14
Cells/mL	Cells per millilitre
cfu/mL	Colony forming units per millilitre
cm	Centimetres
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ESR	Environmental science and research
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FCS	Fetal calf serum
FSC-A	Forward scatter area
FSC-H	Forward scatter height
g	Grams
g	Gravity
GI	Gastrointestinal
h	Hours

H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
I.D	Identification
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN-γ	Interferon gamma
IgA	Immunoglobulin type A
IgE	Immunoglobulin type E
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
IL-10	Interleukin 10
IL-12p40	Interleukin 12 subunit beta
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IκBα	Inhibitor of NF-κB alpha
KCl	Potassium chloride
kDa	Kilodaltons
KH_2PO_4	Potassium dihydrogen phosphate
kPa	Kilopascal
L	Litres
LBP	Lipopolysaccharide binding protein
LTA	Lipoteichoic acid
M	Molar
mA	Milliamps
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight
mCD14	Membrane bound CD14
MD-2	Myeloid differentiation protein 2
MFI	Median fluorescence intensity
mg	Milligrams
min	Minutes

mL	Millilitres
mm	Millimetres
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MRS	de Man, Rogosa and Sharpe (media)
MyD88	Myeloid differentiation primary response gene 88
N	Normality
N.B.	Nota bene
NS	Not significant
N_2	Nitrogen gas
NA	Not applicable
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NEC	Necrotizing enterocolitis
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
ng	Nanograms
NICU	Neonatal intensive care unit
nm	Nanometres
NOD2	Nucleotide binding oligomerization domain 2
NZ	New Zealand
O_2	Oxygen
OD	Optical density
p	Probability
PAF	Platelet activation factor
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononucleocytes
PBS	Phosphate buffered saline
PE	Phycoerythrin

PFA	Paraformaldehyde
pH	Power of hydrogen
PI	Pneumatosis intestinalis
PRRs	Pathogen recognition receptors
psi	Pounds per square inch
R^2	Coefficient of determination
RFI	Relative fluorescence intensity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
rRNA	Ribosomal ribonucleic acid
sCD14	Secreted/soluble CD14
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
spp.	Species plural
SSC-A	Side scatter area
SSC-H	Side scatter height
TEMED	Tetramethylethylenediamine
TFF	Trefoil factor
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth
™	Trademark
U	Units
UK	United Kingdom
USA	United States of America
V	Volts

1.0 Introduction

1.1 Necrotizing enterocolitis

1.1.1 Epidemiology

In 2010, globally, there were approximately 15 million infants born before 37 weeks of gestation[1]. These neonates are defined as premature by the World Health Organisation and are associated with a number of acute health complications such as, but not limited to, necrotizing enterocolitis (NEC)[1]. NEC is a common and severe gastrointestinal (GI) disease that primarily affects premature or very low birth weight infants[2] and results in gut inflammation and intestinal tissue necrosis[3]. It is associated with a high mortality of rate of between 15 and 40%, which is highest in the smallest most immature infants and those requiring surgery[4,5]. The disease is common in neonatal intensive care units (NICUs) and the prevalence is around 4.3% in infants weighing under 1500 g at birth (4342 infants per 100,000 live births), while the total rate in all births is approximately 0.1% (109 infants per 100,000 live births)[5]. Many risk factors have been implicated in NEC, however the most significant risk factor is prematurity[6-8], with smaller infants having an increased incidence and mortality rate[4]. Due to advances in modern medicine, more preterm neonates are surviving birth and early complications[9], and as a result the NEC susceptible population is growing[2,10,11].

Although 90% of NEC cases occur in preterm infants, the disease is also seen in full term neonates. However, the disease risk factors are slightly different and include birth asphyxia, birth defects and low health scores (Apgar scores)[8,12,13]. Typically, increased birth weight results in an earlier onset of NEC[14]. The onset of disease is earlier in term infants (around two days)[15] compared to preterm infants (eight to ten days)[13]. While a few isolated cases of adult NEC-like pathology have been documented, it is extremely rare[16].

1.1.2 Disease characteristics

The ileum and the proximal colon are the most commonly affected sites in cases of NEC, although necrosis can occur anywhere along the length of the gastrointestinal tract[17,18]. NEC results in necrotic intestinal segments where bacteria have translocated across the epithelial barrier.

Pockets of gas, that are bacterial in origin, occur within the intestinal wall (pneumatosis intestinalis (PI)) and also in the abdominal cavity (pneumoperitoneum) and are highly indicative of NEC[19], although NEC may occur without PI[17,20]. After disease onset, infants can succumb to bacteraemia, endotoxemia, sepsis, shock and in severe cases, death[3,8]. Symptoms of NEC include feeding intolerance, abdominal distension, intestinal dysfunction, PI, pneumoperitoneum, hypotension, shock and multiple organ failure[3,21,22]. The disease may progress rapidly, from subtle signs to serious conditions needing immediate medical support within hours[13].

1.1.3 Clinical information

NEC is diagnosed using C reactive protein levels, platelet levels, white blood cell count and abdominal radiographical imaging[13]. Diagnosis of the disease is divided into suspected (stage 1), proven (stage 2) and severe groups (stage 3) (and further divided into subgroups) using a set of rules termed 'Modified Bell's Staging Criteria'[23,24]. The stage is determined by the severity of temperature instability, heart rate, blood platelet decrease, acidosis, PI, portal-venous gas, blood in the stool, abdominal distension, abdominal wall edema, perforated bowel and shock[23,24]. The treatment administered is supportive, depends on the stage of NEC and includes bowel rest, intravenous nutrition, gastric decompression and broad spectrum antibiotics[24]. Surgery is reserved for advanced NEC patients where gut perforation and necrosis has occurred, which may come with its own complications[25]. When surgery is required, resection of the necrotic tissue is necessary via laparotomy, to ensure rapid elimination of contaminated and gangrenous tissue, which would otherwise lead to shock and sepsis[26,27]. Resected bowel due to surgery for the treatment of NEC is a common cause of intestinal strictures, short bowel syndrome, infection and may even affect neurodevelopment[28]. Surgery is associated with the highest mortality rate of NEC infants and NEC may re-occur after surgery[28].

1.1.4 Cause

Although a number of studies have attempted to investigate the pathogenesis of NEC and many risk factors have been identified, no single cause is obvious[3,29,30]. Risk factors that have been implicated in the predisposition of infants to NEC include enteral feeding, formula feeding, hypoxia, intestinal ischemia and also bacterial colonisation[30-32]. However, none are more important than prematurity[6,7].

The gross necrosis seen in NEC is thought to occur after bacterial translocation due to dysregulated barrier function and an inappropriate immune response[19], although it has also been shown that the inappropriate immune response may occur prior to the barrier breach[33]. Undesirable bacterial colonisation of the premature infant is also thought to play a role in the development of NEC[21], while probiotics have been used in an effort to prevent it[23]. It is likely that the cause of NEC is multifactorial[32,34] making it a difficult disease to study, a reason why the pathogenesis is not completely understood. The events and characteristics described here that lead to disease are highlighted in Figure 1.1.

Although many risk factors are strongly associated with NEC, it still remains an important and common disease and the etiology is not entirely understood[3,13,29]. The main contributing factors that specifically relate to the present study will be discussed in further detail.

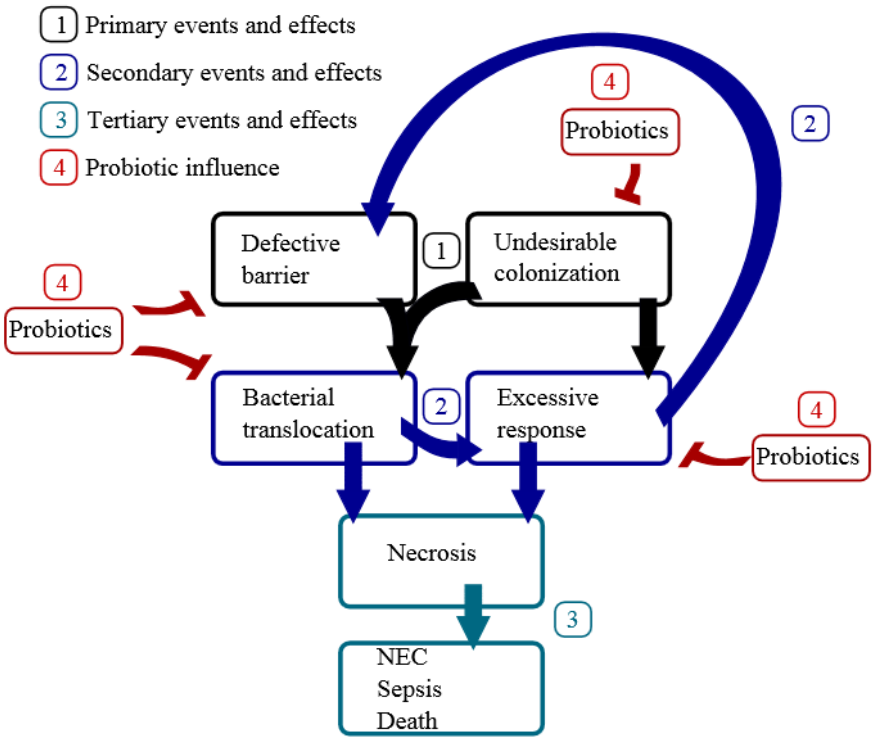


Figure 1.1. The processes that lead to NEC. Infants susceptible to NEC have an undesirable bacterial colonisation pattern and also a defective intestinal barrier (1). These result in bacterial translocation across the mucosal barrier and an inappropriate immune response (2). The ensuing immune response in turn exacerbates the leaky

mucosal barrier (2). Necrosis of the intestinal tissue is caused by bacterial and immune-mediated tissue damage, resulting in gross inflammation and large sections of necrotic bowel tissue appear (3). These are the characteristics of NEC and in severe cases, sepsis and death may occur. It is unknown whether the defective barrier and subsequent bacterial translocation causes the excessive response or whether the initial inflammation drives the defective barrier. Administration of probiotics is hypothesised to have a number of roles that help in the prevention of NEC including improving barrier integrity, preventing undesirable colonisation, reducing bacterial translocation and modulating the immune response (4). Adapted from Lin and Stoll[30] and Hackam et al.[35]

1.2 Intestinal barrier

It is known that preterm infants have a defective intestinal barrier function and it is thought this plays an important role in the development of NEC[21,33,36]. Because 90% of NEC cases occur after feeding, it is thought that the immature gut is prone to intestinal injury and this may be initiated by enteral feeds or bacterial insult[8,37].

1.2.1 Function

The primary role of the gut epithelium is to provide a physical, chemical, immunological and microbial barrier between the body and the luminal contents while allowing for nutrients to be absorbed[38]. The epithelial barrier uses many physical and non-physical components to ensure its integrity. Integral components that help make up the barrier include the epithelial cells and the tight junctions, the mucus layer, peristalsis, immunoglobulins, digestive enzymes, all of which may be impaired in the preterm infant[30,33,39,40]. There are four main cell types that make up the barrier, all with differing but complementing functions. These are the intestinal epithelial cells, goblet cells, Paneth cells and the enteroendocrine cells. The main functions of these cells are absorption and to provide a physical barrier, mucous production, antimicrobial peptide secretion and hormone production respectively[41]. Impairments to the cells or the barrier function can be detrimental to infants and decreased barrier function resulting in bacterial translocation is thought to play a major role in NEC[39].

1.2.2 Gut barrier impairments in NEC

A number of impairments in premature neonates are thought to lead to decreased barrier function and bacterial translocation[42]. "Bacterial translocation is defined as the passage of viable indigenous bacteria from the gastrointestinal tract to extraintestinal sites, such as the mesenteric-lymph-node complex, liver, spleen and bloodstream" and occurs when there is bacterial overgrowth, deficiencies in immunity or there is an increase in permeability to the mucosal barrier[43]. Bacterial translocation is thought to be a major factor in the development of NEC[39].

The enterocytes provide a physical barrier between the luminal contents and the rest of the body and tight junctions surround and join the enterocytes creating a physical barrier to prevent bacterial translocation[30,39]. Immaturity of the tight junctional proteins such as occludin and claudin molecules play a role in the excessive permeability of the premature neonate's mucosal barrier, which may be exacerbated by cytokine production[33]. Proteins associated with tight junctions, such as claudin-3, have been shown to be up-regulated in the urine of NEC-affected neonates, indicating the loss of intestinal barrier integrity[13,44]. Moreover, genes encoding tight junctional proteins including several claudin molecules have been shown to be altered during the early stages of experimental NEC[45], thus implicating defective tight junctions in the pathogenesis of NEC.

Mucus is produced throughout the gut by goblet cells and forms a layer that adheres to the gut epithelium providing lubrication, protection and transport of nutrients. Made up of glycoproteins called mucins, the mucous layer protects the epithelium from direct bacterial adherence and aggregates the bacteria thus enhancing their removal[30,46]. Deficiencies in the mucus lining are thought to be an important contributing factor in NEC, leading to increased bacterial involvement with the mucosal barrier and subsequent bacterial translocation[30]. Mucus composition can change with age, bacterial colonisation and also during mucosal repair, and defects are thought to contribute to NEC induction[33]. In clinical NEC tissues there is a decrease in goblet cells[47] and it is thought that premature neonates have immature goblet cells[30] and a reduced mucus lining[48].

Another important component of the mucus layer is the trefoil factors (TFFs) which are thought to be important in mucosal repair. Also secreted by goblet cells, TFF production is thought to be reduced in the premature gut environment during NEC[49].

IgA is the most common immunoglobulin in the gut mucosa and is a crucial component of the mucosal defence. It is multi-functional and is involved in blocking bacterial adherence and binding potential toxins. IgA is produced in vast amounts in the gastrointestinal tract [50] but its production is decreased in neonates[51,52]. B cells from preterm infant cord blood produce less IgA after a stimulation with a specific B cell stimulating factor (BAFF) compared to B cells from term cord or adult blood[53]. IgM, IgG and IgE are also produced in the GI tract but constitute less than 10% of the antibodies present[50,54]. Deficiencies in secretory IgA in the preterm infant are thought to play a role in the enhancement of bacterial translocation[24]. IgA is also present in breast milk, thereby giving the neonate passive protection from possible pathogens[55] and it is known that breast milk fed infants are less likely to develop NEC[24]. Colostrum, the milk produced within 48 hours of delivery has a higher amount of IgA than normal breast milk[51,56]. Because preterm infants do not often receive colostrum or breast milk, it is thought the lack of passive IgA could be a contributing factor in NEC. However, trials feeding premature infants with IgA supplements have not shown consistent results[57,58].

Gut motility is developmentally regulated and it is known that the immature gut exhibits reduced peristalsis[59]. Gut dysmotility can lead to the luminal contents being in transit for extended periods resulting in increased bacterial proliferation and can cause increased luminal bacteria and antigen exposure[32,42]. The prolonged exposure to the gut contents due to decreased bowel motility in preterm infants also plays an important role in the development of NEC[60,61].

A number of other critical defects in the premature infant gastrointestinal mucosa and environment are also important in the development of NEC, including gastric acidity (less acidic in the premature neonate)[33], low lactase activity, decreased lipase activity, reduced glucose absorption, reduced pepsin production,[62] and also mesenteric ischemia due to the reduction of blood flow in the mucosa[24].

Any combination of the factors highlighted above can perturb the function of the barrier and possibly lead to the translocation of bacteria, a critical step in the development of NEC. Not only is barrier function affected by the maturity of the infant, but also by the bacteria that colonise the host, although colonisation may be a function of prematurity. For the disease to occur, bacteria must be present[60] and therefore the bacterial role in NEC will be discussed next.

1.3 The importance of bacteria in the development of NEC

1.3.1 Bacterial association with NEC

There is compelling evidence to suggest that bacterial colonisation plays a crucial role in the onset of NEC[63]. Firstly, NEC does not occur in the sterile environment *in utero* despite fetal ingestion of proteins, fats and carbohydrates[23,37]. Secondly, NEC only occurs after bacterial colonisation[63-65] and onset of feeding[37]. Other evidence includes the presence of bacteraemia and endotoxemia in NEC infants, PI, outbreaks in certain hospitals, antibiotic and probiotic efficacy and also animal studies (germ-free mice and sanitisation studies), although one single pathogen is unlikely to be the cause[66,67]. Although bacteria and the translocation of bacteria are known to be essential in the process of NEC, it is not known whether the breakdown of the mucosal barrier leads to bacterial translocation, or the bacterial translocation occurs prior to the barrier disruption, and in turn exacerbates it[33]. During NEC, bacteria are thought to be a catalyst in the development of the disease. However, commensal bacteria are also essential for health.

1.3.2 Commensals

There are ten times the number of bacteria residing on or in the human body than there are human cells. The most substantial reservoir of bacteria is our large intestine where up to 10^{11} bacterial cells per gram of faecal content can be present[68,69]. Therefore, the bacterial microflora and its impact on the human host must be significant.

Although some literature suggests that the GI tract contains bacteria prior to birth[70], the neonatal gut is generally regarded as sterile before birth and within minutes, becomes exposed to bacteria through a number of different mechanisms.

Contact with maternal faecal matter during birth, bacteria from the vaginal tract, bacteria from the surrounding environment and also interactions with the mother ensure bacterial colonisation occurs, although it may take years for individuals to show adult microflora compositions[71,72]. There can be between 300 and 500 different species in the adult gut[69], and it is essential to have a healthy gut microflora as it is fundamental in the role of health and disease[73,74]. Common bacterial gut commensals include organisms from four different phyla; Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria[71,75]. Within these phyla, common genera that inhabit the gastrointestinal tract include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus*[76,77]. However, during early colonisation it is thought that the infantile gut is almost exclusively colonised by *Bifidobacterium* spp.[71]. Commensals are important inhabitants of the gut for a number of reasons.

Effects of commensal bacteria include nutritional (vitamin synthesis and carbohydrate fermentation) and anti-pathogenic (competing for pathogen attachment sites, reducing bacterial translocation which can result in sepsis, blocking enteroinvasive species, competing for available nutrition and producing growth inhibiting substances)[69]. Other essential roles carried out by commensals include educating the immune system via 'controlled inflammation' (a process where commensals induce a small amount of healthy "physiological" inflammation in order to induced the development of immune cells), increasing the epithelium vasculature, improving the development of the gut-associated lymphoid tissues, reducing inflammation, increasing immunoglobulin levels, stimulating the development of the enteric nervous system, stimulating IL-10 production, strengthening tight junctions and enhancing gut motility[42,68,69].

It is obvious that commensal bacteria are crucial in the normal development of infants. However, it is thought that preterm and NEC-affected infants have altered colonisation patterns, and unfavourable bacteria may become established. Therefore the role that undesirable microbes play in the development of NEC will be discussed further.

1.3.3 NEC-associated bacteria

It has been demonstrated that the infant gut is predominantly colonised by Bifidobacteria during the early stages of life[71]. However, it has been shown that preterm infants have a different colonisation pattern than that of full term infants[78,79] and that this may play a role in the development of NEC[80].

The composition of the microflora is very important during development, and deficiencies of beneficial commensal bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp., are observed in underweight neonates[81,82]. The bacteria that colonise the gut of neonates are impacted upon by the route of birth, diet and the presence of antibiotics[69]. The microbiota obtained after a caesarean section differs from those born naturally, and characteristically show a delayed colonisation and significantly fewer *Escherichia coli*[83].

Diet can also change the colonising bacteria. Many preterm infants are fed formula milk instead of breast milk and it is thought that *Enterococcus* and *Bacteroides* spp. predominate in formula fed infants while breast fed infants are dominated by *Bifidobacterium* species[71,78,84]. Antibiotics are often used in NICUs, in order to control and prevent bacterial infections, and can delay beneficial bacterial colonisation and select for pathogenic or antibiotic resistant strains. Prolonged antibiotic use is associated with an increased incidence of NEC[61,79]. However, it is not only the microbial composition that is important, the order in which bacterial species are acquired termed 'succession' is also important for gut health[72,85].

Unfavourable colonisation my lead to the establishment of unwanted bacteria and NEC has been associated with a number of bacterial species which include bacteria from the genera *Escherichia*, *Salmonella, Klebsiella*, *Enterobacter*, *Clostridium* and also *Staphylococcus*. Although many members of these genera can be thought of as commensals, their overgrowth may cause an inappropriate immune response or result in bacterial translocation, especially in the hyperactive immature gut environment and in turn contribute to NEC[86,87].

The family of bacteria, *Enterobacteriaceae* has previously been implicated in NEC cases[6,33,88,89], and interestingly, this family of bacteria are the most frequently found to translocate through the mucosal barrier, with Gram positives and obligate anaerobes translocating less frequently[43]. Specific species including *Cronobacter sakazakii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* have been implicated in NEC cases or outbreaks[90-93] and some have been shown to bind to epithelial cells and induce apoptosis and cell injury without invasion[94]. *C. sakazakii* in powdered milk formula has been linked to outbreaks of NEC in hospitals and it has been shown to exacerbate disease in mouse models of NEC, implicating it in the disease[93,94]. Also, it has been found that immature or NEC-affected infants are often enriched in Gammaproteobacteria[33,79] while exhibiting less diversity in terms of the total gut microflora overall[79,82]. Moreover, it is noted that the diversity of bacterial species present in infants fed formula milk is vastly different to those fed breast milk, possibly leading to a higher risk of disease[84,95]. NEC-affected babies, and also premature babies in general, exhibit significantly decreased diversity in bacterial species in faecal samples, as detected by culture methods and 16S rRNA[79,82]. NEC-affected babies were significantly enriched in Proteobacteria and had a decrease in other bacterial phyla. Also, disease was associated with an increased length of antibiotic course. It was also apparent that each individual affected by NEC was often predominantly colonised by a single species, as opposed to multiple species in healthy controls[79].

A drawback of some studies includes the fact that the diversity of bacteria was analysed after NEC diagnosis. Therefore, the question arises whether the unfavourable bacteria present are involved in initiating NEC or whether they occur after it. However, another study demonstrated, by 16S rRNA analysis, that there were more Proteobacteria and Actinobacteria and fewer *Bifidobacterium* spp. and *Bacteroides* spp. in infants who went on to develop NEC and these differences were no longer apparent at the time of diagnosis. The same study documented an increase in *K. pneumoniae*, *Klebsiella granulomatis*, *Staphylococcus epidermidis* and *Clostridium perfringen*s in NEC cases, although no single species was thought to be the sole cause[96].

Although colonisation patterns are different in preterm and NEC-affected infants and certain bacterial families are implicated in the disease, it is apparent that no single entity is the cause[33]. In light of this information, it is apparent that NEC probably occurs due to the function of multiple bacteria and not necessarily a single pathogen, and that an incorrect colonisation pattern of the preterm gut influenced by certain factors such as antibiotics, feeding and other factors contributes to NEC[79].

1.3.4 Probiotic bacteria

Probiotic bacterial supplementation has been investigated in the prevention of NEC as pre-term infants have a lack of desirable bacteria such as *Bifidobacterium* and *Lactobacillus* species[23,81,97,98]. Probiotics are defined as "…living microorganisms that upon ingestion in specific numbers, exert health benefits beyond those of inherent basic nutrition" and by definition, do not necessarily have to colonise to exert their effects[69].

Probiotics are known to have a variety of effects on the gut environment including producing antimicrobial agents, reducing bacterial translocation, reducing mucosal permeability, maintaining epithelial cell tight junctions, increasing immunoglobulin and mucous levels, regulating the pH of the luminal contents, regulating apoptosis, reducing inflammatory molecules such as cytokines, chemokines and nitric oxide and also helping to reduce the adherence of possibly harmful bacteria[60,99].

Bacteria that have been used as probiotic agents in an effort to reduce the incidence of NEC include *Lactobacillus acidophilus, Bifidobacteria infantis, Streptococcus thermophilus, Bifidobacterium bifidum and Lactobacillus reuteri* with varying results, although some promising decreases in disease risk have been demonstrated [59,81,98,100,101]. Infloran®, a specific prophylactic treatment for NEC that contains live *B. infantis* and *L. acidophilus,* has shown a decrease in NEC incidence and severity in a number of studies, although its mechanism is not well known[81,102]. It is hoped that with the introduction of probiotics, a reduction in the use of antibiotics can be achieved[80]. It is important to investigate thoroughly all species of probiotics as the effects of one strain cannot be extrapolated to another even if they are the same species[69]. Furthermore, it is essential to recognise that bacteria often have multiple effects on the host and therefore caution should be taken[81].

It must be understood that these premature infants are among the most at risk, frail individuals and a delicate, informed and logical approach is needed. The fact still remains that the complete pathophysiology of NEC is unclear and therefore treatment or prevention of the disease is also imperfect[60]. Although probiotic administration has been shown to be effective in reducing the incidence of NEC, and many theories for the mode of action have been hypothesised, the exact mechanism or mechanisms of the mode of action of probiotics in NEC are not fully understood[102,103]. One hypothesis for the mode of action of probiotics in NEC is that of immunomodulation[104].

1.3.5 Bacteria and immune function

The gut-associated lymphoid tissue has the largest reservoir of immunocompetent cells in the body and the development of the gut immunity is regulated by the intestinal microflora[69,73]. Germ-free animals have defects in the development of gut-associated lymphoid tissue, develop fewer and defective Peyer's patches, produce less IgA, and also fewer lamina propria associated T cells, while the wider immune system is also affected[69,105]. Attenuation of inflammatory responses caused by pathogens can occur with the aid of commensals[42] and in general, commensals stimulate tolerance and down regulate the immune system while pathogens cause an increase in responsiveness[106]. The anti-inflammatory contribution of probiotics is thought to be achieved in part by down-regulating cytokine, chemokine and other pro-inflammatory molecule production, possibly mediated by inhibition of the transcription factor NF-κB and through the action of the inhibitor of NF-κB, IκBα[104,107,108].

In diseases such as inflammatory bowel disease (IBD), treatment with certain genera of bacteria, including bifidobacteria, lactobacilli and *Bacteroides* spp., have been shown to have a number of immunoregulatory functions such as IL-10 induction, regulatory T cell generation, TNF-α and iNOS reduction, reduced NF-κB activation and decreased IL-8, IL-12p40 and IFN-ɣ gamma levels[73]. However, it is apparent that these effects are achieved by certain signals from bacteria that remain largely unknown.

1.4 Gut immune function

1.4.1 Immunity

Directly beneath the epithelial barrier reside specialised immune cells that are able to initiate immune responses. These include macrophages, dendritic cells, T cells and B cells. In addition to this, specialised epithelial cells, termed microfold cells (M cells), overlay follicles termed Peyer's patches. These cells continuously sample the luminal contents, presenting antigen to underlying immune cells. Dendritic cells are able to protrude through epithelial cells, directly assessing the luminal contents[41]. Immune activation requires the detection of pathogens, achieved by the recognition of pathogen-associated molecular patterns (PAMPs) by host immune pattern recognition receptors (PRRs). An important set of PRRs includes the toll like receptors (TLRs) and a common PAMP that is recognised is LPS[109].

During the recognition of Gram negative bacteria via endotoxin, LPS is bound to a soluble lipopolysaccharide binding protein (LBP) and subsequently brought to CD14. From here it is passed to the myeloid differentiation protein 2 (MD-2) and interacts with a TLR4 dimer[110]. Signalling cascades (highlighted in Appendix 5.2, Figure 5.14) are activated that may be MyD88-dependent or independent and result in NF-κB activation and translocation and in turn a number of inflammatory genes are expressed[111].

Despite the presence of specialized immune cells directly beneath the epithelial layer and their obvious important roles in gut homeostasis and infection, it is thought that the immunological function of the intestinal epithelial cells is crucial in the development of NEC[112,113].

1.4.2 Enterocyte immune function

Enterocytes not only provide a physical barrier but also play a role in immune function and it is known that IECs are able to produce a number of immunomodulatory factors[35,114]. TLRs are expressed by enterocytes[113] and the ability of the intestinal epithelial layer to respond to bacteria first involves the recognition of bacteria using these molecules[41]. There are many different TLRs present on and within human cells, however an important TLR that is often implicated in NEC is TLR4[33,67].

1.4.3 TLR4, CD14 and NEC

TLR4 is known to be required for the recognition of LPS[110,115]. Also, TLR4 expression on enterocytes has been implicated in the induction of NEC. Firstly, TLR4 is increased in human and mouse tissues that are affected by NEC[22,37,116]. Secondly, a villin-cre knockout system that specifically removes TLR4 from enterocytes in a mouse model of NEC showed a significant decrease in NEC induced cell damage indicated by the autophagy marker LC3[112]. It has also been shown that TLR4 mutant mice are protected from experimental NEC and that TLR4 activation leads to an increase in gut epithelial cell apoptosis and reduced IEC migration, suggesting that TLR4 activation may decrease mucosal repair and barrier function[22]. Also, TLR4 expression is up regulated in the immature gut environment compared to the fully mature intestine, leading to an exaggerated inflammatory response[29,60,61]. In some diseases, cellular TLR4 distribution can change depending on patient disease status and differences in TLR4 localisation are seen between inflammatory bowel disease patients and healthy controls[117]. The TLR4 complex can localise to the apical or basolateral cell surface of differentiated intestinal epithelial cells[117,118] indicating that luminal and translocated LPS can be detected.

The expression of TLR4 on IECs is known, however the expression of CD14 (the co-receptor for LPS) on epithelial cells is debatable[119-121]. It has previously been shown that CD14 is important for certain cells to respond to LPS (primary monocytes and macrophages and cell lines)[122,123] and that CD14 can increase the responsiveness or binding of certain cells to endotoxin[39,124]. CD14 is implicated in NEC and has been shown to be increased in NEC-affected tissue in humans and mice[37,120]. Specifically, the expression of CD14 on enterocytes is thought to be important in the induction of NEC[120]. The increased expression of these molecules may also be due to factors associated with NEC, such as LPS exposure and hypoxia[22,120], and it has previously been shown that stimulation of enterocytes with LPS can cause an increase in CD14 and TLR4 expression *in vitro* and *in vivo*[120,125]. Furthermore, changes in TLR4 mRNA and protein expression levels as well as increased cytokine production in a rat model of NEC have been found to occur 48 hours prior to the histological damage to the intestinal barrier, indicating the immune response may lead to the damage seen in NEC[126].

Monoclonal CD14 blocking antibodies or LPS neutralizing peptides were used to alleviate disease in mouse models, implicating endotoxin and the co-receptor in the pathology of NEC[37]. CD14 is known to exist in multiple forms and some studies propose that soluble CD14 (sCD14) is produced by enterocytes and is important for epithelial cell responsiveness to LPS[119,127]. However, other studies have found membrane bound CD14 (mCD14) on IECs[29,120].

Genetic polymorphisms in the form of single nucleotide polymorphisms (SNPs) of TLR4 and CD14 are not thought to play a role in the predisposition of infants to NEC[128]. Also, mutations to the nucleotide binding oligomerization domain 2/ caspase activation and recruitment domain 15 (NOD2/CARD15) gene are not thought to play a role in NEC predisposition[129]. Furthermore, common SNP mutations in cytokine and chemokine genes have not been associated in the susceptibility of infants to NEC[130]. Moreover, infantile twin studies have shown that NEC may occur in one twin and not the other, putting genetic susceptibilities in doubt[79,131].

In light of this, it is evident that gut epithelial cells not only act as a physical barrier to infection, but can also act immunologically. It is apparent that the expression of TLR4 and CD14 on enterocytes may be important in the development of the disease. Furthermore, genetic mutations in genes encoding proteins with immune functions are not important in the predisposition of NEC. Recognition of bacteria is important, and the response elicited is what drives the immune function.

1.4.4 Interleukin 8

It is known that enterocytes are able to produce a wide range of immune modulators including cytokines and chemokines[132]. Inflammatory molecules that have been implicated in the pathogenesis of NEC include TNF-α, interleukins 1, 6, 8, and 12, nitric oxide and also platelet activating factor (PAF)[31,133] and it is evident that levels of circulating[134,135] and localised[26] inflammatory factors are increased.

An important pro-inflammatory factor implicated in NEC is IL-8[63,135]. IL-8 acts on neutrophils enhancing their migration to sites of inflammation and also helping with their activation[136].

These cells are known to produce a variety of pro-inflammatory and destructive factors including reactive oxygen species, lytic enzymes, macrophage inflammatory proteins, TNF-α, IFN-γ, IL-1β, and IL-8[137,138]. Intestinal injury caused by neutrophil migration and activation has been linked to the pathogenesis of NEC[21,139] and it is known that inflammatory molecule release by neutrophils can cause ischemia, tight junction disruption and barrier permeability, all of which are important components in NEC. This implicates IL-8, the neutrophil chemo-attractant in the initiation of the disease[21,39,139].

IECs are capable of producing large amounts of IL-8 in response to LPS, bacteria or inflammatory cytokines[140,141] and, compared to control infants, IL-8 levels are known to be significantly higher in NEC-affected tissue and serum[21,135,142]. Also, it has been previously demonstrated that stimulation of IECs with different species and even strains of bacteria can cause differential pro-inflammatory cytokine and IL-8 expression[141]. This gives rise to the possibility that the discrepancy in bacterial colonisation patterns between premature and term infants leading to increased pro-inflammatory signalling may contribute to the predisposition to NEC.

It is thought that the immature gut epithelium elicits an inappropriate IL-8 production after stimulation and it has been shown that fetal cell lines have an exaggerated LPS mediated IL-8 response compared to adult intestinal epithelial cells[21,63,143]. Furthermore, it has been shown that the production of IL-8 by intestinal epithelial cells after a pro-inflammatory challenge is reduced when probiotics are introduced[144,145], suggesting a possible mechanism for the reduction in NEC by probiotics. Anti-inflammatory effects of a *Lactobacillus* species in a mouse model of colitis were linked to a decrease in the recruitment of neutrophils[146]. This implicates probiotics in the reduction of neutrophil recruitment, a process that may be beneficial during NEC. Probiotics, even when non-viable, are known to have a number of immunomodulatory effects and factors that have been shown to affect immune function. These include probiotic DNA, lipoproteins, cell wall components and cytoplasmic components[147]. The Gram positive cell wall component lipoteichoic acid (LTA) has many immunomodulatory effects[148,149] and is known to have pro-inflammatory effects on innate immune cells[150]. However, in IECs it has been shown that LTAs from probiotic bacteria are able to reduce the IL-8 production in response to LPS[151].

Also, LTA has been shown to interact with the LPS co-receptor CD14[152,153], possibly competing with LPS binding. This anti-inflammatory effect by LTA may provide a mechanism by which probiotics can decrease mucosal inflammation and possibly account for the reduced incidence of NEC.

The fact that the immature gut is predisposed to a response that leads to an inappropriate IL-8 production, in turn promoting neutrophil emigration and activation suggests a role of IL-8 in NEC.

1.5 Summary

As discussed, there are a number of contributing factors associated with NEC. Current evidence suggests a multifactorial mechanism that includes intestinal immaturity, colonisation of unfavourable bacteria, reduction of important commensals, barrier dysfunction, IEC apoptosis, decreased restitution, hyper-responsiveness to LPS, increased TLR4 and CD14 molecules, IL-8 up regulation, neutrophil recruitment, inflammatory molecule production, bacterial translocation and subsequent necrosis[3,22,29,30,120]. This multifactorial process is summarized in Figure 1.2. It is the aim of this study to further investigate the pathogenesis of NEC and the role of probiotics. This study defines the role of the production of IL-8 by enterocytes during NEC in response to LPS and various strains of NEC-associated and clinically isolated *Enterobacteriaceae* using HT-29 and Caco-2 cells as model IECs. The ability of clinically implicated probiotic bacteria isolated from Infloran® to act in an immunoregulatory manner in a pro-inflammatory environment using LPS and the *Enterobacteriaceae* was also investigated. Moreover, we assessed the role of TLR4 and CD14 expression in enterocytes and LPS signalling.

Our underlying hypothesis is: Unfavourable bacterial colonisation causes an exaggerated pro-inflammatory response, characterised by IL-8 production, by enterocytes in the premature neonate. This leads to inappropriate neutrophil recruitment, barrier dysfunction and permeability. Subsequent bacterial translocation further exacerbates the inflammation and disease progression, while probiotics may reduce the undesirable bacterial colonisation, translocation or the pro-inflammatory response.

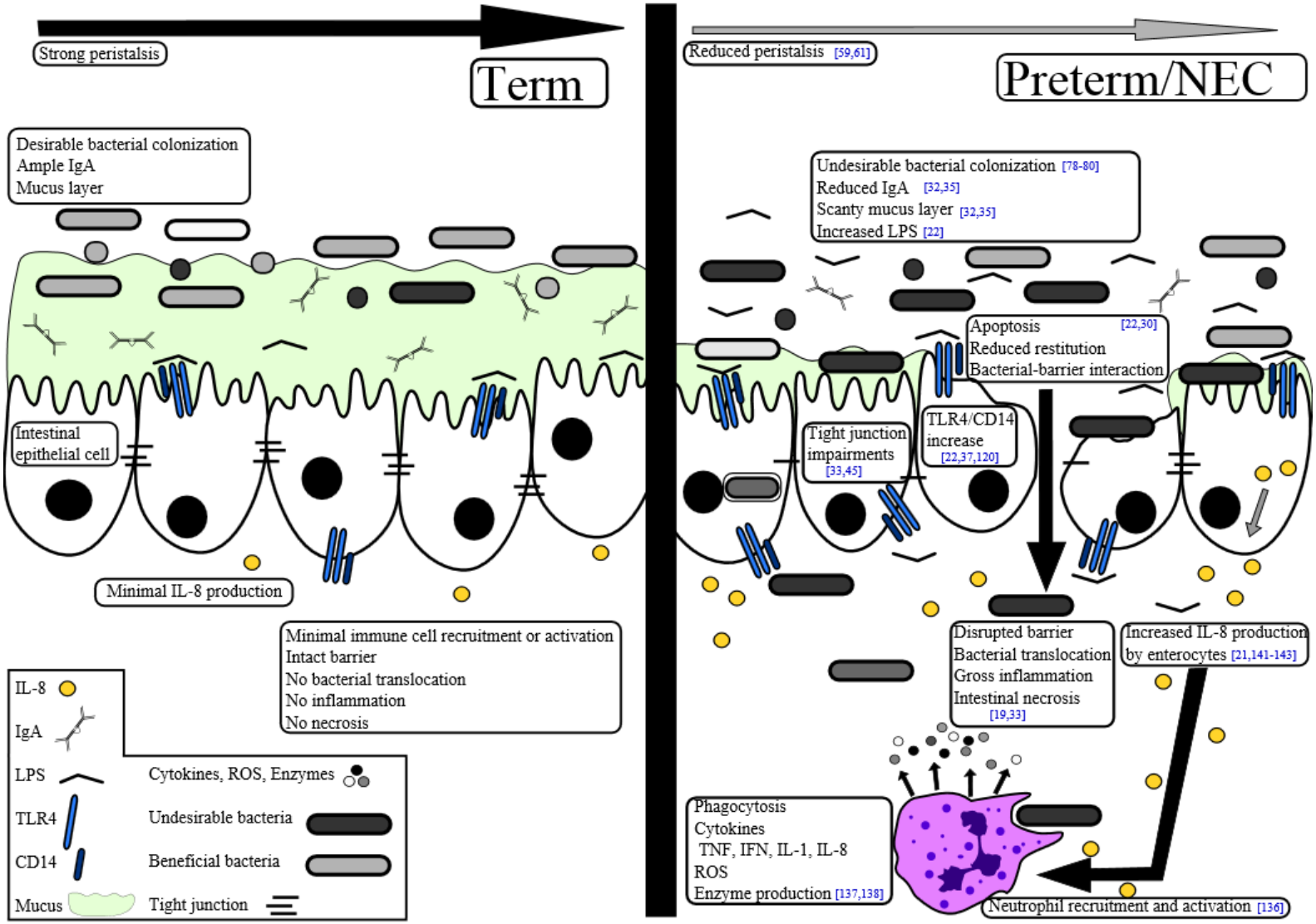

Strong peristalsis
Term
Reduced peristalsis [59,61]
Preterm/NEC
Desirable bacterial colonization
Ample IgA
Mucus layer
Intestinal epithelial cell
Minimal IL-8 production
Minimal immune cell recruitment or activation
Intact barrier
No bacterial translocation
No inflammation
No necrosis
Undesirable bacterial colonization [78-80]
Reduced IgA [32,35]
Scanty mucus layer [32,35]
Increased LPS [22]
Apoptosis [22,30]
Reduced restitution
Bacterial-barrier interaction
Tight junction impairments [33,45]
TLR4/CD14 increase [22,37,120]
Disrupted barrier
Bacterial translocation
Gross inflammation
Intestinal necrosis [19,33]
Increased IL-8 production by enterocytes [21,141-143]
Phagocytosis
Cytokines
TNF, IFN, IL-1, IL-8
ROS
Enzyme production [137,138]
Neutrophil recruitment and activation [136]
IL-8
IgA
LPS
TLR4
CD14
Mucus
Cytokines, ROS, Enzymes
Undesirable bacteria
Beneficial bacteria
Tight junction

Figure 1.2. Factors and processes in the premature/NEC susceptible neonate that are thought to contribute to NEC. The preterm neonate GI tract has a number of critical defects that may contribute to NEC. Firstly; the mucus coat, GI peristalsis and IgA concentration are reduced in the premature neonate, which allows for increased antigen, toxin and bacterial association with the mucosal barrier. Secondly, preterm infants have an undesirable bacterial composition which is in part responsible for certain defective components of the mucosal barrier. The undesirable bacteria also facilitate inflammation via increased levels of immune stimulating factors such as LPS. Apoptosis, reduced restitution and tight junctional protein impairments allow for the passage of luminal contents such as bacteria, LPS and immunostimulatory antigens through the mucosal barrier. Increased TLR4 and CD14 expression in the preterm gut are thought to contribute to the inappropriate inflammatory response seen during NEC that is characterised by excessive cytokine and chemokine production. IL-8 production causes neutrophil migration and activation, enabling the production of factors such as cytokines, chemokines, ROS and enzymes, all of which increase the inflammation and contribute to the disruption of the mucosal barrier. The presence and translocation of bacteria (especially *Enterobacteriaceae* species) compounds the inflammatory response and tissue damage and cell death occurs. These processes are essential contributors that lead to the gross necrosis of the intestinal wall and excessive inflammation during NEC. Translocation of bacteria results in sepsis and endotoxemia, while in advanced cases a cytokine storm may ensue possibly followed by death.

1.6 Aims

The aims of this study are based on the hypothesis that LPS and/or *Enterobacteriaceae* induce a pro-inflammatory response from enterocytes by their interaction with the CD14/TLR4 complex. Subsequent IL-8 production leads to an increased inflammatory response possibly initiating the pathology of NEC due to barrier disruption and bacterial translocation. The effects of purified LPS and a number of *Enterobacteriaceae* that have been isolated from infants with NEC (Table 1.2) or implicated in NEC outbreaks (*C. sakazakii* strains, Table 1.2) were tested on the human colonic epithelial cell line HT-29 and the IL-8 production was measured. Probiotic species from Infloran® (*Bifidobacterium infantis* and *Lactobacillus acidophilus*) used clinically in the Dunedin NICU and also purified LTA was examined for their ability to influence the effect LPS and NEC-associated bacteria have on IL-8 production by the HT-29 cells.

The specific aims of this study are:

I. To examine the effect of purified LPS and also NEC-associated bacteria on the production of IL-8 by HT-29 and Caco-2 cells using ELISA analysis.

II. To test whether bacterial isolates from different NEC group infants (control, suspected or confirmed) influence IL-8 production.

III. To determine the effect of probiotic bacteria and purified LTA on IL-8 production by HT-29 cells and their ability to influence IL-8 production in response to purified LPS of NEC-associated bacteria.

IV. To assess the contribution to pro-inflammatory signalling in HT-29 cells by CD14 and TLR4.

V. To determine the effect of endotoxin on TLR4 and CD14 expression in HT-29 cells in order to deduce whether this contributes to NEC.

2.0 Materials and methods

2.1 Media preparation

All media was prepared and stored according to the manufacturers' instructions. Liquid media used for aerobic bacterial culture was 3% (30 g/L) tryptic soy broth (TSB), (Bacto™, Becton, Dickinson and Company (BD), USA) pH to 7.3 ± 0.2. For anaerobic bacteria pre-reduced 5.5% (55 g/L) Lactobacili MRS broth (MRS) (Difco™, BD) pH to 6.5 ± 0.2 was used. Sterile broths were obtained by autoclaving bacterial media in glass universals at 121°C, 15 psi (103 kPa) for 15 min. Dulbecco's Modified Eagle Medium with D-glucose, L-glutamine, sodium pyruvate and phenol red (DMEM) (Gibco®, Life Technologies, USA), 10% foetal calf serum (FCS) (BioInternational, New Zealand) and 0.015% 10,000 U/mL penicillin with 10,000 µg/mL streptomycin (Gibco®, Life Technologies), (diluted 1:100 in DMEM), was used for HT-29 and Caco-2 cell culture (complete DMEM), while complete DMEM supplemented with 0.055 mM 2-mercaptoethanol (2-ME) (Gibco®, Life Technologies) was used for THP-1 cell culture. Roswell Park Memorial Institute media 1640 with L-glutamine (RPMI) (Invitrogen™, Life Technologies, USA), with 10% FCS, and 0.015% 10,000 U/mL penicillin with 10,000 µg/mL streptomycin (diluted 1:100 in RPMI) and 0.055 mM 2-ME (Complete RPMI) was used for PBMC cell culture.

Solid media used included 3% TSB with 1.5% (15 g/L) bacteriological agar (TSA) (Coast Biologicals Ltd, Auckland, NZ) pH to 7.3 ± 0.2 for aerobic bacteria and pre-reduced 5.5% MRS broth with 1.5% bacteriological agar pH to 6.5 ± 0.2 for anaerobic bacteria. Five percent MacConkey Agar (Difco™, BD) with 1.5% bacteriological agar pH to 7.1 ± 0.2 was used in the identification of *Enterobacteriaceae*. Agar was autoclaved at 121°C, 15 psi (103 kPa) for 15 min and poured into plastic Petri dishes 90 mm x15 mm (LabServe®, Thermo Fisher, NZ).

Table 2.1 Media and culture conditions used routinely for bacterial and cell culture

Cell type/organism	Description	Media	Culture Conditions (37°C)
Aerobic Bacteria	Bacteria	3% Bacto™ TSB/TSA	Aerobic
Anaerobic Bacteria	Bacteria	5.5% Difco™ MRS broth/agar	Anaerobic 0% O_2, 16% CO_2
Human HT-29 ATCC: HTB-38	Human colorectal adenocarcinoma (epithelial cell)	Complete DMEM	Aerobic, 5% CO_2
Human Caco-2 ATCC: HTB-37	Human colorectal adenocarcinoma (epithelial cell)	Complete DMEM	Aerobic, 5% CO_2
Human THP-1 ATCC: TIB-200	Human acute monocytic leukemia (monocyte)	Complete DMEM + 0.055 mM 2-ME	Aerobic, 5% CO_2
Human PBMCs	Human peripheral blood mononuclear cell	Complete RPMI	Aerobic, 5% CO_2

2.2 Bacterial strains

Enterobacteriaceae strains used were previously isolated from infant faecal samples from infants in the Dunedin Hospital NICU[6] or purchased from the ESR culture collection. Probiotic bacteria were previously isolated from Infloran® (Swiss Serum and Vaccine Institute, Berne, Switzerland). All bacterial species used during the present study are highlighted in Table 2.2.

Table 2.2 Source of aerobic and anaerobic bacterial strains used in this study

Type	Organism	Isolate I.D/strain	NEC infant status	Source
Aerobic organisms:	***K. pneumoniae***	III 4	Control	NICU
	K. oxytoca	V 6	Control	NICU
	K. oxytoca	V 7	Control	NICU
	E. coli	V 3	Control	NICU
	S. marcescens	IV 9	Suspected	NICU
	K. oxytoca	VIII 1	Suspected	NICU
	R. ornithinolytica	XV11 1	Suspected	NICU
	K. pneumoniae	VIII 8	Suspected	NICU
	K. oxytoca	I 3	Confirmed	NICU
	K. oxytoca	I 4	Confirmed	NICU
	K. oxytoca	I 11	Confirmed	NICU
	E. cloacae	I 1	Confirmed	NICU
	C. sakazakii	50	NA	ESR
	C. sakazakii	2029	NA	ESR
Anaerobic organisms:	***B. infantis***	-	NA	Infloran®
	L. acidophilus	-	NA	Infloran®

2.3 Aerobic bacterial culture

Aerobic bacteria (Table 2.2) glycerol or bead stocks were inoculated into TSB or TSA, incubated overnight at 37°C and subsequently cultured onto TSA. Colony morphologies were recorded, Gram stains performed, growth and lactose fermentation ability on MacConkey agar was checked, API 10 or API 32 E rapid strips (Biomerieux, USA) conducted and overnight cultures of bacteria sent to the Southern Community Laboratories, Dunedin for matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis to confirm bacterial identities. Cultures were stored at 4°C when not in use and isolates were subcultured at least twice before use in experiments.

2.4 Anaerobic bacterial culture

A single bead from *Bifidobacteria infantis* or *Lactobacillus acidophilus* (Table 2.2) bead stocks was inoculated into MRS broth or agar, and incubated for 48 h at 37°C in anaerobic conditions using an AnaeroPack system (Mitsubishi Gas Chemical Company, Tokyo, Japan). A loop or single colony was subsequently cultured onto pre-reduced MRS agar. Colony morphologies were recorded and Gram stains undertaken to confirm bacterial identities. Cultures were stored at 4°C when not in use and isolates were subcultured at least twice before use in experiments.

2.5 Bacterial growth curves

C. sakazakii 2029, *C. sakazakii* 50, *E. cloacae* I 11 and *K. pneumoniae* VIII 8 were inoculated into 35 mL of sterile TSB for growth curves and incubated at 37°C with shaking (Innova™ 4000, New Brunswick Scientific, USA). Five millilitre samples were taken at 1, 2, 3, 4, 5, 6 and 24 h after inoculation. Each sample was centrifuged at 5000 rpm (Digifuge GL, Heraeus-Christ, Germany) for 10 min and pellets resuspended in 5 mL PBS (Appendix 5.2). One millilitre of each suspension was read at 600 nm (Novaspec II, Pharmacia Biotech, USA), referenced with PBS. Tenfold serial dilutions in sterile TSB of each sample were made at each time point, ranging from $1x10^{-3}$ to $1x10^{-7}$, and spread or dropped in duplicate or quadruplicate onto TSA in 100 µL or 10 µL amounts respectively then incubated 37°C overnight. Results are indicative of three independent experiments.

Colonies were counted and cfu/mL were calculated using the formula:

$$cfu/mL = N(1/V)D)$$

Where N = number of colonies, V = volume plated and D = dilution factor

2.6 Doubling dilutions

(I) Aerobic bacteria (Table 2.2). Each strain was inoculated into 20 mL of sterile TSB and incubated at 37°C aerobic conditions for 24 h with shaking. A 2 mL sample was taken after 24 h and doubling dilutions to 1:256 were made, in sterile TSB and read at 600 nm, referenced with sterile TSB. Tenfold serial dilutions in sterile TSB of each sample were also made ranging from $1x10^{-3}$ to $1x10^{-7}$, and dropped in 10 µL amounts in quadruplicate onto TSA and incubated at 37°C for 24 h. Colonies were counted and cfu/mL was calculated using the previous formula.

(II) Anaerobic bacteria. Each strain (Table 2.2) was inoculated into 35 mL of sterile MRS broth and incubated at 37°C anaerobic conditions for 48 h. A 5 mL sample was taken at 48 h and doubling dilutions were made as outlined above in sterile MRS broth and read at 600 nm, referenced with sterile MRS broth. Tenfold serial dilutions in sterile MRS of each sample were also made as outlined above and spread or dropped in duplicate or quadruplicate onto MRS agar in 100 or 10 µL amounts respectively and incubated at 37°C for 48 h. Colonies were counted and cfu/mL was calculated using the previous formula. Results are representative of three individual experiments.

2.7 Heat inactivation of bacteria

(I) Aerobic bacteria. Overnight cultures (Table 2.2) were inoculated into 20 mL or 35 mL TSB and incubated at 37°C with shaking for 24 h. Optical densities and bacterial counts were carried out as previously described. Cultures were incubated at 80°C with shaking for 20 min. A loop or duplicate 10 µL amounts from each culture was spread or dropped onto TSA and incubated overnight at 37°C to check heat inactivation was successful. Heat-killed bacteria were centrifuged at 5000 rpm for 10 min (Digifuge GL, Heraeus-Christ) or 4000 rpm for 10 min (Heraeus™, Multifuge™ X3R, ThermoScientific, USA) and pellets washed in 5 mL PBS. A final centrifugation was conducted, supernatants were discarded and cells resuspended in 5 mL of complete DMEM, aliquoted and stored at -20°C until required.

(II) Anaerobic bacteria. Forty-eight hour cultures of *B. infantis* and *L. acidophilus* (Table 2.2) were inoculated into 20 mL MRS broth and incubated at 37°C anaerobic conditions for 48 h. Optical densities and bacterial counts were carried out as previously described. Cultures were then incubated at 80°C with shaking for 120 min. A loop from each culture was spread onto MRS agar and incubated for 48 h at 37°C in anaerobic conditions to ensure no viable bacteria remained. Heat-killed bacteria were then centrifuged, washed and stored as above.

2.8 Human cell culture and cell lines

All cell culture techniques were carried out in a class II biological safety cabinet (Herasafe, KS, ThermoScientific) with sterile reagents and ethanol (70%) sterilized equipment. Cell lines were stored in 1 mL of their corresponding complete media with 10% DMSO (AnalaR, England) at $1x10^6$ cells/mL in vapour phase of liquid nitrogen. HT-29 (HTB-38, ATCC, USA) human colorectal adenocarcinoma epithelial cells and Caco-2 (HTB-37, ATCC) human colorectal adenocarcinoma epithelial cells were resuscitated and maintained at 37°C with 5% CO_2 in 50 mL or 250 mL tissue culture treated flasks (BD Falcon™, USA) with 6 mL or 12 mL complete DMEM respectively. Complete DMEM + 0.55 mM 2-ME was used to resuscitate and maintain THP-1 (TIB-202, ATCC) human acute monocytic leukemia monocyte cells, while complete RPMI was used for PBMC culture. Media was changed every 4-5 days, and when 70-80% confluence was reached, adherent cells were treated with 2 mL trypsin EDTA (Gibco®, Life Technologies™) diluted in sterile PBS or 2 mL TrypLE (Gibco®, Life Technologies™) for approximately 7 min. Cells were collected and spun at 300 x *g* 5 min at room temperature (Heraeus™, Multifuge™ X3R, ThermoScientific) and subcultured in complete media at $1x10^5$ cells/mL in a 250 mL flask for continued culture or $3x10^5$ cells/mL in a 24 or 96 well tissue culture treated plate (BD Falcon™) for assays. THP-1 cells were differentiated into adherent THP-1 macrophages for assays by the addition of 5 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich®, USA) for 24 h. HT-29 cells, PMA treated THP-1 cells and PBMCs were allowed to adhere for 24 h before any experimental manipulations were undertaken, while Caco-2 cells were allowed to adhere for 1, 6 or 16 days into non-, partially or completely differentiated cells respectively. Cells were used within 10 passages from resuscitation.

A haemocytometer (Boeco, Germany) or automated cell counter (Countess®, Invitrogen™, Life Technologies, USA) and 0.2% trypan blue (Invitrogen®, Life Technologies) were used for all cell counts and viability assays.

2.9 HT-29 stimulation for IL-8 production

HT-29 cells were seeded at $3x10^5$ cells/mL into a 96 well plate and incubated for 24 h in 37°C and 5% CO_2. For IFN-γ prime or co-stimulation assays, cells were either primed with 100 µL of 10 ng/mL human IFN-γ (BioLegend®, USA) for 12 h prior to LPS (Sigma-Aldrich®) stimulation, or incubated with 10 ng/mL IFN-γ along with the 24 h LPS stimulation. Supernatants were removed and cells were subsequently stimulated with 100 µL of 1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL or 0 ng/mL of LPS diluted in complete DMEM or with $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL (MOI of 3.3, 33.3 and 333.3 respectively) of heat-killed bacteria from Table 2.2 and diluted in complete DMEM in duplicate. Supernatant samples were taken at 6, 8, 20 and 24 h time points and stored at -20°C.

2.10 Enzyme linked immunosorbent assays (ELISAs)

A full list of antibodies used during this study can be found in Table 2.3.

IL-8 ELISA kit (OptEIA™, BD Biosciences, USA) was used according to manufacturer's instructions. Ninety-six well ELSIA plates (MaxiSorp®, Nunc, Denmark) were coated with 50 µL anti-human IL-8 monoclonal capture antibody (OptEIA™) diluted 1:250 in coating buffer (Appendix 5.1) sealed and incubated at 4°C overnight. Plates were washed five times with approximately 300 µL wash buffer (Appendix 5.1) per well and blocked at room temperature for 1 h with 200 µL of assay diluent (Appendix 5.1) and washed as above. IL-8 standards were conducted using doubling dilutions of lyophilized human IL-8 (OptEIA™) from 200 pg/mL, to 3.1 pg/mL and also 0 pg/mL in assay diluent, run in duplicate and parallel with each assay. Samples were centrifuged at 300 x *g*, (Centrifuge 5810 R, Eppendorf, Germany or Heraeus™, Multifuge™ X3R, ThermoScientific) for 10 min in 96 well plates and diluted 1:50 in assay diluent prior to adding to ELISA plate.

Fifty microlitre amounts of standards and samples were added to corresponding wells, plate sealed and incubated for 2 h at room temperature and then washed again as above. Fifty microlitres of working detector containing biotinylated anti-human IL-8 monoclonal detection antibody (OptEIA™) 1:250 in assay diluent and streptavidin-horseradish peroxidase conjugate (SAv-HRP) enzyme reagent (OptEIA™) 1:250, was added to each well. The plate was sealed and incubated for 1 h at room temperature. A final wash step was conducted as described above and 50 µL of tetramethylbenzidine (TMB) with hydrogen peroxide (BD Biosciences, USA) substrate solution was added and plate incubated at room temperature in the dark for 30 min. Fifty microlitres of 1 M H_2SO_4 stop solution was added to each well and the plate was read immediately at 450 nm with a 550 microplate reader (Bio-Rad, USA). IL-8 concentrations were determined from the standard curve. Briefly, this was carried out by plotting absorbance values against the known IL-8 concentrations of the standard curve and applying the subsequent equation to all experimental absorbance values. Experiments with standard curves containing a line of best fit R^2 value of less than 0.95 were discarded and ELISA repeated. All experimental absorbance values were checked to ensure they lay within the dilution curve.

2.11 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from 40 mL of whole blood from a healthy subject. The University of Otago Human Ethics Committee approved human blood research (Ref# 12/036), and all subjects involved gave informed consent. Whole blood was obtained by a professional phlebotomist (Southern Community Laboratories, Dunedin) into 10 mL green top heparinized plasma tubes (Vacutainer®, BD, USA). In a class II biological safety cabinet, whole blood was diluted 1:1 in PBS in 20 mL amounts and overlaid gently onto 15 mL Ficoll-Paque PLUS (GE Healthcare, UK). Ficoll-blood overlay was spun at 800 x *g*, no brake at room temperature for 20 min (Centrifuge 5810 R, Eppendorf), buffy coat was removed gently by Pasteur pipette and added to 10 mL complete RPMI. Cells were spun at 1700 rpm, 4°C, for 4 min with the brake on. Supernatants were removed and cells were resuspended in 20 mL complete RPMI. At this point a 10 µL aliquot was taken and cell number and viability were assessed as indicated previously (section 2.8).

Cells were seeded into a 250 mL falcon flask at $1x10^6$ cells/mL and incubated for 2 h, 37°C, 5% CO_2. Supernatants were discarded and cells were washed twice gently with complete RPMI and incubated for 24-48 h in 20 mL complete RPMI 37°C, 5% CO_2. Supernatants were discarded and cells incubated with 2 mL PBS based, enzyme free, cell dissociation buffer with EDTA (Gibco®, Life Technologies) for 15 min. Cells were then scraped (BD Falcon™) and added to 50 mL tubes (BD Falcon™) and the flask washed with complete RPMI to ensure all cells were gathered and spun at 1700 rpm, 4°C, brake on for 4 min. Supernatants were removed and cells were then either stained for flow cytometry analysis as described for HT-29 cells (2.12), resuspended at $1x10^6$ cells/mL in FACS buffer (Appendix 5.1) for flow cytometric titration and treated as outlined (2.12), resuspended at $1x10^6$ cells/mL in sterile PBS and processed for Western blotting as described for HT-29 cells (2.13), or resuspended at $1x10^6$ cells/mL in complete RPMI with 10% DMSO and stored in liquid N_2 (section 2.3).

2.12 Flow cytometry analysis

HT-29 or Caco-2 cells were seeded at $1x10^6$ cells/mL into a 24 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were discarded and cells were subsequently stimulated with 1 mL of 1000 ng/mL, 100 ng/mL 10 ng/mL, 1 ng/mL or 0 ng/mL of LPS diluted in complete DMEM and incubated for 24 h. Supernatants were discarded and cells were dislodged using 1 mL PBS based, enzyme free, cell dissociation buffer with EDTA (Gibco®) incubated at room temperature for 15 min and cells aspirated vigorously.THP-1 cells were seeded at $1x10^6$ cells/mL into a 24 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were discarded and cells were subsequently differentiated into adherent macrophages with 1 mL 5 ng/mL PMA diluted in complete DMEM + 2-ME, or left untreated for 24 hours. For adherent THP-1 or PBMC macrophages, supernatants were discarded and cells were dislodged using 1 mL PBS based, enzyme free, cell dissociation buffer with EDTA (Gibco®) incubated at room temperature for 15 min and aspirated vigorously. For non-adherent THP-1 cells, supernatants were collected and aspirated. Dislodged cells were added to FACS tubes (BD Falcon™) and wells washed and aspirated with FACS buffer. Viability and cell numbers were assessed as described in section 2.14. Cells were centrifuged (Centrifuge 5810 R, Eppendorf) at 300 x *g*, 4°C, 5 min and supernatants were discarded.

The remaining pellets were resuspended in 1 mL FACS buffer by vortexing. Three hundred microlitre amounts of cell suspensions were transferred to fresh FACS tubes, and centrifuged as above. Supernatants were discarded and when a live/dead stain was incorporated, cells were resuspended in 1 mL of live/dead stain (Live/Dead® Fixable Red Dead Cell Stain, Life Technologies™), diluted 1:1000 in FACS buffer. Cells were vortexed and incubated for 30 min in the dark on ice. Cells were subsequently vortexed, 1 mL FACS buffer added, vortexed again and spun as above. Supernatants were then discarded and when an FcR block was used, cells were resuspended in residual FACS buffer along with 5 µL of Human TruStain FcX (Fc Receptor Blocking Solution, BioLegend) and incubated on ice for 20 min in the dark, followed by a further centrifugation as previous and resuspended in 100 µL of FACS buffer. Direct cell surface staining was achieved by the addition of 1-5 µL of PE mouse anti-human CD14 antibody (Clone: M5E2, BD Pharmingen, USA) or 2.5 µL of mouse anti-human CD14 brilliant violet (Clone: M5E2, BioLegend). Isotype controls included were IgG2a-κ PE isotype control (Clone: eBM2a, eBioscience, USA), rat IgG2a-κ PE isotype control (Clone: eBR2a, eBioscience), mouse IgG-κ PE isotype control (Clone: MOPC-21, BioLegend) or brilliant violet mouse IgG2a κ isotype control antibody (Clone: MOPC-173, BioLegend), in 1-5 µL amounts for PE isotypes or 2.5 µL for the brilliant violet isotype. Conversely, for CD14 titration on PBMCs, after being treated as outlined above for HT-29 cells, PBMCs were resuspended in 100 µL of FACS buffer along with 0 µL, 0.1 µL, 0.5 µL, 1 µL, 2.5 µL, 5 µL or 10 µL of anti-CD14 brilliant violet mouse anti-human CD14 (M5E2, BioLegend) or brilliant violet mouse IgG2a κ isotype control (MOPC-173, BioLegend). Cells were incubated in the dark on ice for 30 min, resuspended in 1 mL of FACS buffer by vortex and subsequently centrifuged as above. Supernatants were discarded and cells resuspended in 200 µL 2% paraformaldehyde (PFA), (Sigma-Aldrich®) and stored at 4°C in darkness for later analysis. To remove PFA before analysis, 1 mL of FACS buffer was added and vortexed and cells centrifuged as above, discarding supernatant in PFA discard. Cells were resuspended in 200-300 µL FACS buffer and analysed on an LSR Fortessa Flow Cytometer (BD Biosciences). The flow cytometry analysis software FlowJo_V10 (FlowJo, TreeStar, USA) was used to analyse flow data. At least 10,000 events were recorded for each treatment during each individual experiment.

Side and forward scatter criteria was used to identify the HT-29 cell population and remove debris and doublets. Live/Dead Red fluorescence was used to exclude dead cells while PE or brilliant violet fluorescence identified CD14 expression. Minimal spectral overlap was observed, therefore compensation was not used. Gating strategies for flow cytometry plots can be found in Appendix 5.2, Figure 5.7.

2.13 TLR4 and CD14 Western blot

HT-29 cells were seeded at $3x10^5$ cells/mL into a 24 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were removed and cells were stimulated with 1 mL 1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL or 0 ng/mL LPS diluted in complete DMEM and incubated for 24 h. Supernatants were removed and 0.5 mL PBS based, enzyme free, cell dissociation buffer with EDTA (Gibco®) was added and adherent cells were scraped, all contents were collected into microfuge tubes and spun at 4000 rpm (Heraeus™, Sepatech, Biofuge™ 13, Germany) for 10 min. Supernatants were discarded, pellets resuspended in 1 mL PBS and spun as above. Supernatants were again discarded and pellets resuspended in 55 µL Western blot sample buffer (Appendix 5.1) diluted 1:1 in PBS and incubated at room temperature for 30 min. Samples were stored at -20°C until required. Directly prior to western blotting, samples were boiled for 10 min and between 10-15 µL of each sample was added to each well in a 10% SDS-PAGE gel (Appendix 5.1) and run for 70-80 min at 170 V, 250 mA (AE-6500 Dual Mini Slab, Atto, Japan) in electrophoresis running buffer (Appendix 5.1). Protein quantification and equal protein loading was not carried out as preliminary experiments showed equal actin protein amounts. A molecular weight marker (PageRuler Plus Prestained Protein Ladder, ThermoScientific) was added to estimate band size. Using the semi-dry method (Trans-Blot® Semi-Dry Transfer Cell , Bio-Rad), proteins were transferred to a nitrocellulose membrane (Hybond C extra, GE Healthcare) at 20 V, 250 mA for 20 min using appropriate buffers (Appendix 5.1). Membranes were blocked with 0.1% sodium caseinate-alanate 180 (NZMP, New Zealand) for 60 min and nitrocellulose membranes were placed in 50 mL falcon tubes.

Proteins were detected using mouse anti-human CD14 (1:500, Clone: MEM-18, Abcam®, UK) or mouse anti-human TLR4 (1:1000, Clone: 25, Santa Cruz Biotechnology, USA) and goat anti-human actin (1:2000, Clone: I-19, Santa Cruz Biotechnology), incubated in 3 mL PBS with 10 µL Tween-20 (Sigma-Aldrich®) at 4°C with rocking overnight for CD14, or for 60 min, room temperature with rocking for TLR4 or actin. Membranes were washed with agitation for 4 x 5 min in PBS with 0.01% Tween-20. CD14 and TLR4, primary antibodies were detected with donkey anti-mouse, 800 CW (1:20,000, IRDye®, Li-Cor®, USA). Actin was detected with donkey anti-goat, 680 RD (1:20,000, IRDye®, Li-Cor®). Antibodies were incubated for 60 min with rocking at room temperature in the dark. Membranes were washed as above in the dark, and then soaked for 5 min in PBS. Fluorescent signals were detected by exposing membrane at 700 nm for 2 min and 800 nm for 10 min on an Odyssey Fc Western blot infrared detector (Li-Cor®). Protein signal strength was determined with Image Studio™ Software (Li-Cor®).

Table 2.3 Antibodies and their applications and concentrations

Application	Antibody	Concentration	Use
Flow cytometry	PE mouse anti-human CD14 (Clone: M5E2, BD Pharmingen)	1-5 µL per 100 µL	CD14 primary
	Brilliant Violet mouse anti-human CD14 (Clone: M5E2, BioLegend)	2.5 µL per 100 µL	CD14 primary
	PE mouse IgG2a-κ isotype control (Clone: eBM2a, eBioscience)	1-5 µL per 100 µL	Isotype control
	PE rat IgG2a-κ isotype control Clone: (eBR2a, eBioscience)	1-5 µL per 100 µL	Isotype control
	PE mouse IgG-κ isotype control (Clone: MOPC-21, BioLegend)	1-5 µL per 100 µL	Isotype control
	Brilliant Violet IgG2a-κ isotype control (Clone: MOPC-173 BioLegend)	2.5 µL per 100 µL	Isotype control

	Human TruStain FcX (Fc Receptor Blocking Solution, BioLegend)	5 µL per 100 µL	FcR block
Flow cytometry titration	Brilliant Violet mouse anti-human CD14 (Clone: M5E2, BioLegend)	0, 0.1, 0.5, 1, 2.5, 5 or 10 µL per 100 µL	CD14 on PBMCs
	Brilliant Violet IgG-κ isotype control (Clone: MOPC-173, BioLegend)	0, 0.1, 0.5, 1, 2.5, 5 or 10 µL per 100 µL	Isotype control on PBMCs
ELISA analysis	BD OptEIA anti-Human IL-8 Capture antibody	1:250	Capture antibody
	BD OptEIA anti-Human IL-8 biotinylated antibody	1:250	Detection antibody
Western blot	Mouse anti-human CD14 (Clone: MEM-18, Abcam®)	1:500	CD14 primary
	Mouse anti-human TLR4 (Clone: 25, Santa Cruz Biotechnology)	1:1000	TLR4 primary
	Goat anti-human actin (Clone: I-19, Santa Cruz Biotechnology)	1:2000	Actin primary
	Donkey anti-mouse, 800 CW (IRDye®, Li-Cor®)	1:20,000	CD14/TLR4 secondary
	Donkey anti-goat, 680 RD (IRDye ®, Li-Cor®)	1: 20,000	Actin secondary
***in vitro* block**	Mouse anti-human CD14 (Clone: MEM-18, Abcam®)	5, 2.5, 1.2,0.6, 0.3 or 0.1 µg/mL	CD14 block
	Mouse anti-human TLR4 (Clone: 25, Santa Cruz Biotechnology)	5, 2.5, 1.2,0.6, 0.3 or 0.1 µg/mL	TLR4 block

2.14 Probiotics and IL-8 production

HT-29 cells were seeded at $3x10^5$ cells/mL into a 96 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were removed and cells were incubated with 100 µL of $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL heat-killed *B. infantis* or *L. acidophilus* diluted in complete DMEM for a variety of times prior to the addition and 24 hour incubation of 100 µL of $1x10^7$ heat-killed *C. sakazakii* 2029, *C. sakazakii* 50, *E. cloacae* I 1 or *K. pneumoniae* VIII 8 or 100 ng/mL LPS, diluted in complete DMEM. Alternatively, cells were incubated with 100 µL of $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL heat-killed or live *B. infantis* or *L. acidophilus* alone or with 100 ng/mL LPS or $1x10^7$ cfu/mL heat-killed *C. sakazakii* 50 for indicated lengths of time in 37°C and 5% CO_2. The equations generated during the dilution curves (Figure 3.7) were used to calculate live probiotic concentrations in cfu/mL from an OD read. Supernatants were removed and stored at -20°C until ELISA analysis.

2.15 Effect of CD14 and TLR4 antibody block on IL-8 production

HT-29 cells were seeded at $3x10^5$ cells/mL into a 96 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were removed and cells were washed with 100 µL sterile PBS and incubated with 100 µL of doubling dilutions of mouse anti-human CD14 (Clone: MEM-18, Abcam®) or mouse anti-human TLR4 (Clone: 25, Santa Cruz Biotechnology) from 5 µg/mL to 0.16 µg/mL in complete DMEM and incubated for 1, 3 or 6 h prior to the addition of 100 ng/mL LPS or for 24 h, along with the addition of 50 ng/mL LPS. Supernatants were stored at -20°C until ELISA analysis.

2.16 Effect of LTA on IL-8 production

HT-29 cells were seeded at $3x10^5$ cells/mL into a 96 well plate and incubated for 24 h in 37°C and 5% CO_2. Supernatants were removed and cells were washed with 100 µL sterile PBS and incubated with 100 µL of tenfold serial dilutions of *Streptococcus faecalis* (now *Enterococcus hirae* in Sigma® database) LTA (Sigma-Aldrich®) from 100 µg/mL to 0.01 µg/mL in complete DMEM and incubated for 24 h alone or with 100 ng/mL LPS diluted in complete DMEM. Supernatants were stored at -20°C until ELISA analysis.

2.17 Statistical analysis

Results were analysed and graphed using GraphPad Prism 6 statistical software (GraphPad Software Incorporation, USA) with the assistance of Dr. Josie Athens (Department of Preventive & Social Medicine, University of Otago). Statistical analysis was conducted on data with three or more biological replicates. p values of 0.05 or less were considered significant, as indicated.

2.18 Diagram design

Diagrams were created using information from indicated references using the software; Inkscape, version: 0.48.4 (Software Freedom Conservancy, USA).

3.0 Results:

3.1 IL-8 production by IECs

3.1.1 Production of IL-8 in response to LPS by HT-29 cells

Enterocytes have been implicated in the immune response during NEC, specifically in their ability to produce IL-8 after a pro-inflammatory stimulation[21]. Therefore the ability of enterocytes to produce IL-8 after a stimulation with LPS was investigated.

HT-29 cells were exposed to various concentrations of LPS for 6, 8, 20 or 24 hours (Figure 3.1 a and b). A significant ($p < 0.05$) increase in IL-8 production compared to the untreated control was found in response to 10, 100 and 1000 ng/mL LPS. For all following experiments, 100 ng/mL LPS was used as an IL-8 positive control, as it elicited the strongest response. The greatest significant difference ($p < 0.0001$) in IL-8 production was seen after 24 h (Figure 3.1 b), therefore all subsequent stimulation assays continued for 24 h. The ability of IFN-γ to increase LPS induced IL-8 production was also tested. However, a significant increase in IL-8 production compared to LPS alone was not found (Figure 3.1 c and Appendix 5.2, Figure 5.1). Therefore an IFN-γ co-stimulation or pre-incubation was not used in subsequent experiments.

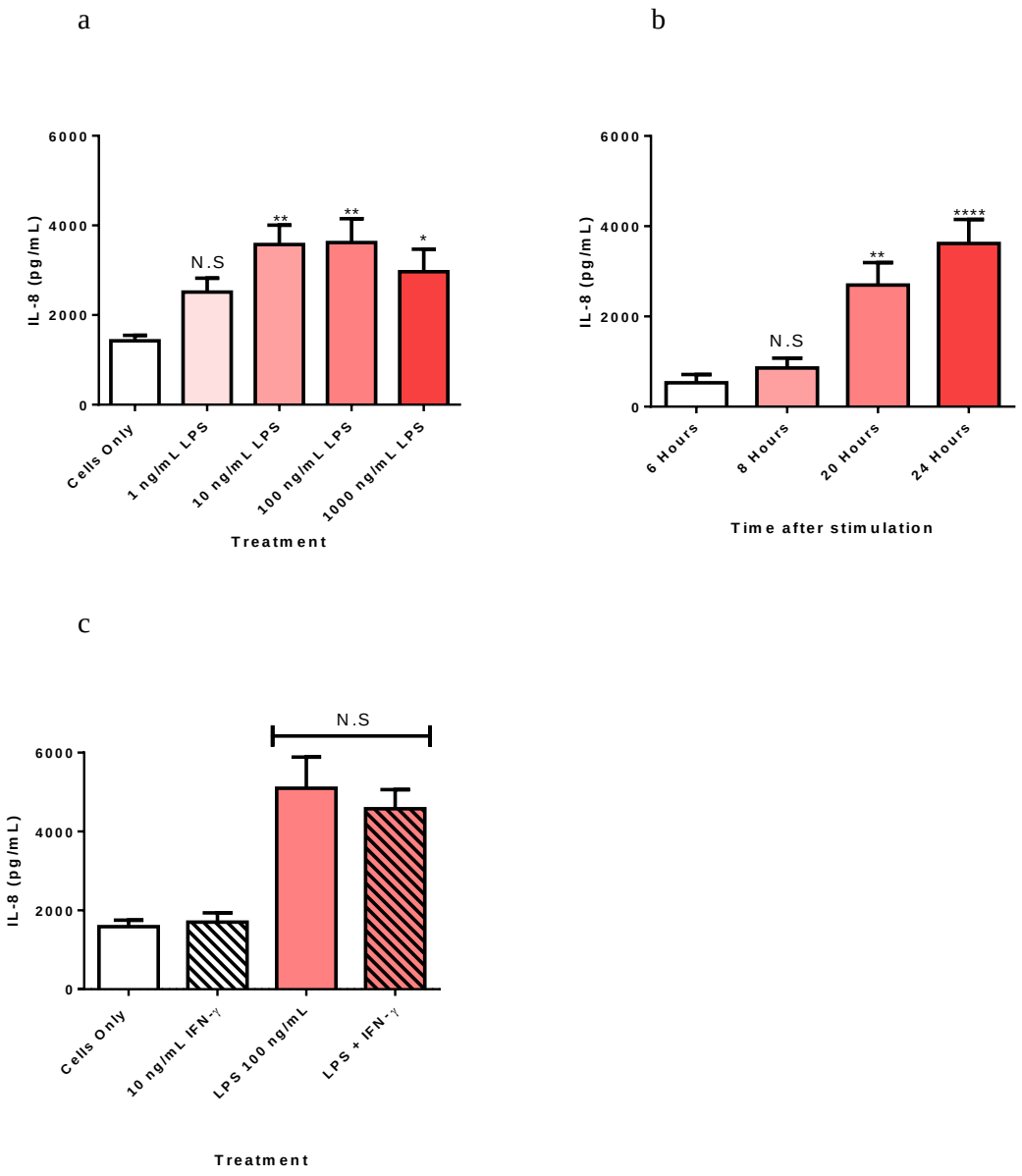
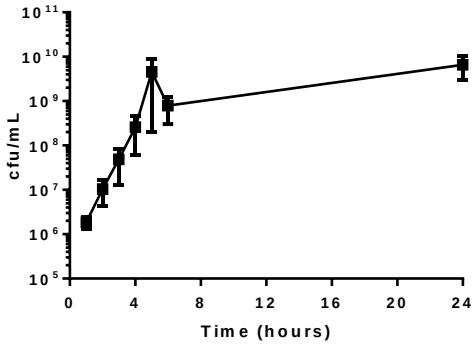


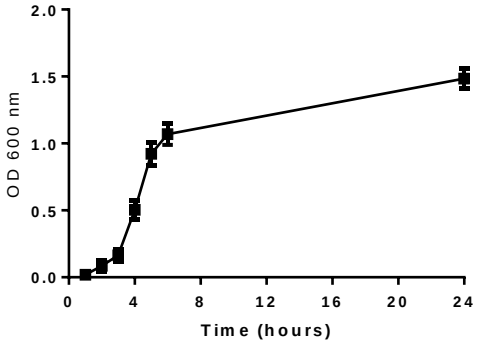
Figure 3.1. IL-8 production by HT-29 cells in response to bacterial LPS. Semi-confluent HT-29 cells were incubated for 24 h with 0, 1, 10, 100 or 1000 ng/mL *E. coli* LPS (a) or for 6, 8, 20 or 24 hours with 100 ng/mL *E. coli* LPS (b). Statistical analysis; one way ANOVA with multiple comparisons (Dunnet's multiple comparison test). N.S, not significant;* $p < .05$; ** $p < 0.01$ and **** $p < 0.0001$. Bars and error bars represent the pooled mean and the SEM of duplicate wells of three separate experiments. Semi-confluent HT-29 cells were co-incubated with 10 ng/mL IFN-γ and 100 ng/mL LPS for 24 hours (c). Statistical analysis; one way ANOVA with multiple comparisons (Tukey's multiple comparison test). N.S, not significant. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

3.1.2 Growth of NEC-associated bacteria

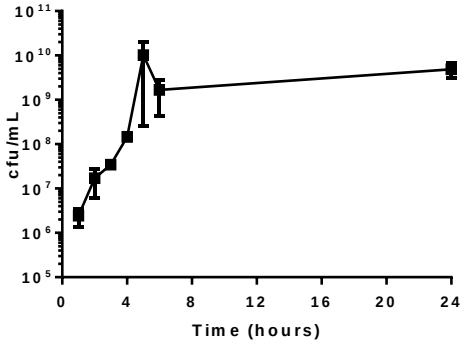
For NEC to occur, bacteria must be present[154] and it is known that enterocytes are able to produce inflammatory molecules (predominantly IL-8) in response to certain stimuli[155], including bacteria[145,156]. Prior to the stimulation assay, the growth pattern of four NEC-associated bacteria was determined. API 10 rapid tests were used to confirm the identity of each bacterium. Twenty-four hour growth curves were conducted and optical densities read for each of the four bacteria at various time points (Figure 3.2). *C. sakazakii* 50, *C. sakazakii* 2029 and *K. pneumoniae* VIII 8 reached approximately $5x10^9$ cfu/mL after 24 h while *E. cloacae* I 1 reached approximately $1x10^9$ cfu/mL after 24 h. For each isolate, a brief lag phase was observed between 0 and 2 hours after inoculation, as indicated by the optical densities. Exponential growth for all organisms stopped after approximately 5 h and stationary phase continued for the duration of the time course. The growth characteristics of these bacteria allows us to estimate bacterial numbers from the optical densities for future experiments and know what growth phase bacteria are in.

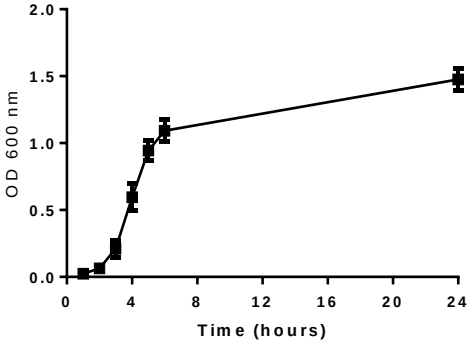
C. sakazakii 50



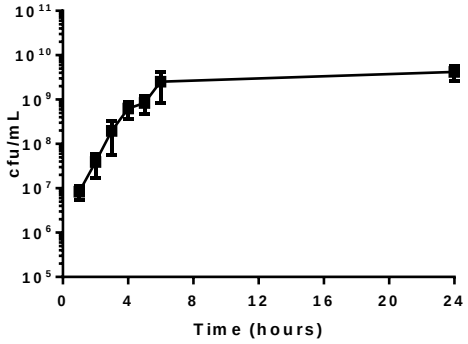


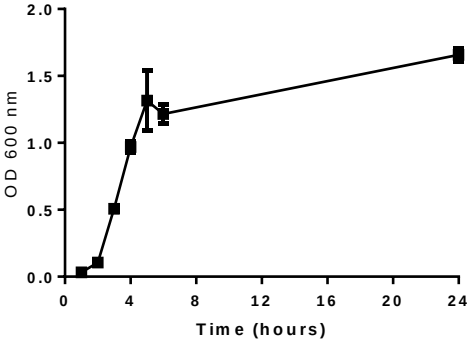
C. sakazakii 2029





K. pneumoniae VIII 8





E. cloacae I 1

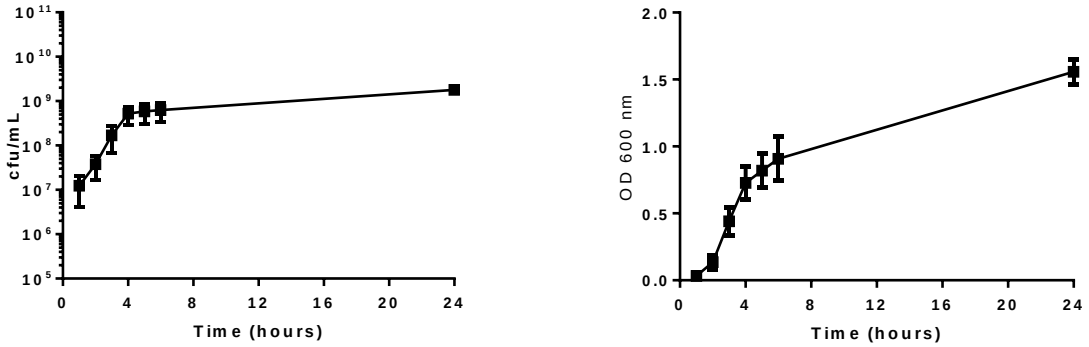
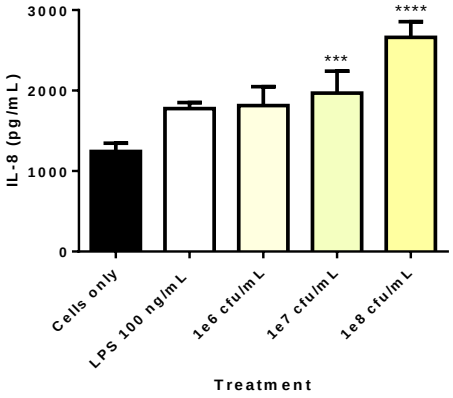


Figure 3.2. Growth characteristics of four NEC-associated bacteria. *C. sakazakii* 50, *C. sakazakii* 2029, *K. pneumoniae* VIII 8 and *E. cloacae* I 1 were incubated for 24 h, 37°C with shaking in TSB and sampled at indicated time points. Colony forming units (left column) were determined using tenfold serial dilutions. Optical densities (right column) of the same four isolates were read at 600 nm. Data points and error bars represent the pooled mean and the SEM of three separate experiments.

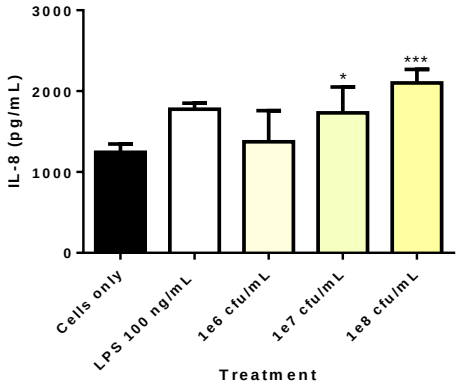
3.1.3 IL-8 response of HT-29 cells in the presence of heat inactivated bacteria

With the growth characteristics of the four bacterial strains determined, the IL-8 production by HT-29 cells in response to varying concentrations of the four heat-killed, NEC-associated bacteria was measured. The IL-8 production by HT-29 cells was significantly ($p < 0.05$) increased in response to $1x10^7$ and $1x10^8$ cfu/mL of the four bacterial strains after 24 hours (Figure 3.3 a-d). The increase in IL-8 production was time-dependent with the highest amount of IL-8 produced after 24 h (Figure 3.4 a-d). Caco-2 cells are another intestinal epithelial cell line and are able to be differentiated into polarized cells that express microvilli over time. Similar results were obtained from partially (6 day adherence) and completely (16 day adherence) differentiated Caco-2 cells, where $1x10^7$ cfu/mL *C. sakazakii* 50 and also various concentrations of LPS stimulated IL-8 production after 24 hours (Figure 3.5 b and c). It has previously been shown that Caco-2 cells are able to produce IL-8, at similar quantities obtained in the current study, in response to a 24 hour LPS stimulation[63]. Very little IL-8 production was observed in non-differentiated (24 hour adherence) Caco-2 cells (Figure 3.5 a). In contrast, LPS and NEC-associated bacteria did not induce TNF-α, IL-1β, IL-6 or IL-10 production by HT-29 cells (Appendix, Figure 5.15). For bacterial heat-kill, *Enterobacteriaceae* were cultured for 24 hours and then heated at 80°C for 20 minutes as highlighted in section 2.7.

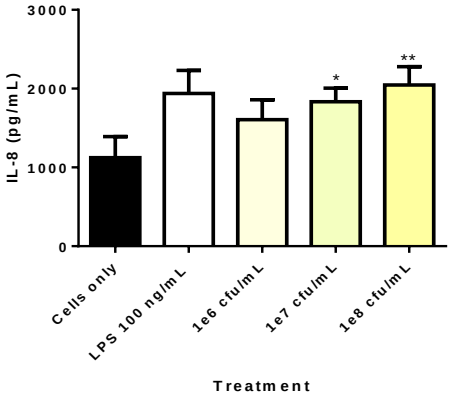
a *C. sakazakii* 50



b *C. sakazakii* 2029



c *K. pneumoniae* VIII 8



d *E. cloacae* I 1

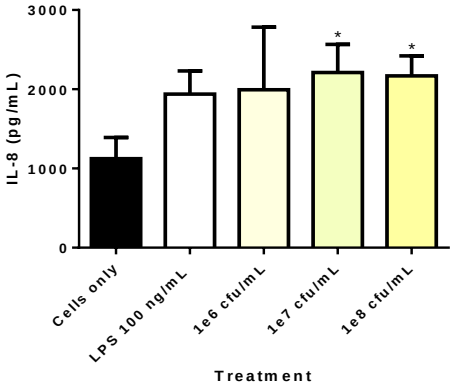


Figure 3.3. IL-8 production by HT-29 cells in response to four strains of heat inactivated NEC-associated bacteria. Semi-confluent HT-29 cells were incubated for 24 h with $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL of washed, heat-killed *C. sakazakii* 50, *C. sakazakii* 2029, *K. pneumoniae* VIII 8 or *E. cloacae* I 1(a-d). Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Statistical analysis; one way ANOVA with multiple comparisons (Dunnet's multiple comparison test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared to the cells only control. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

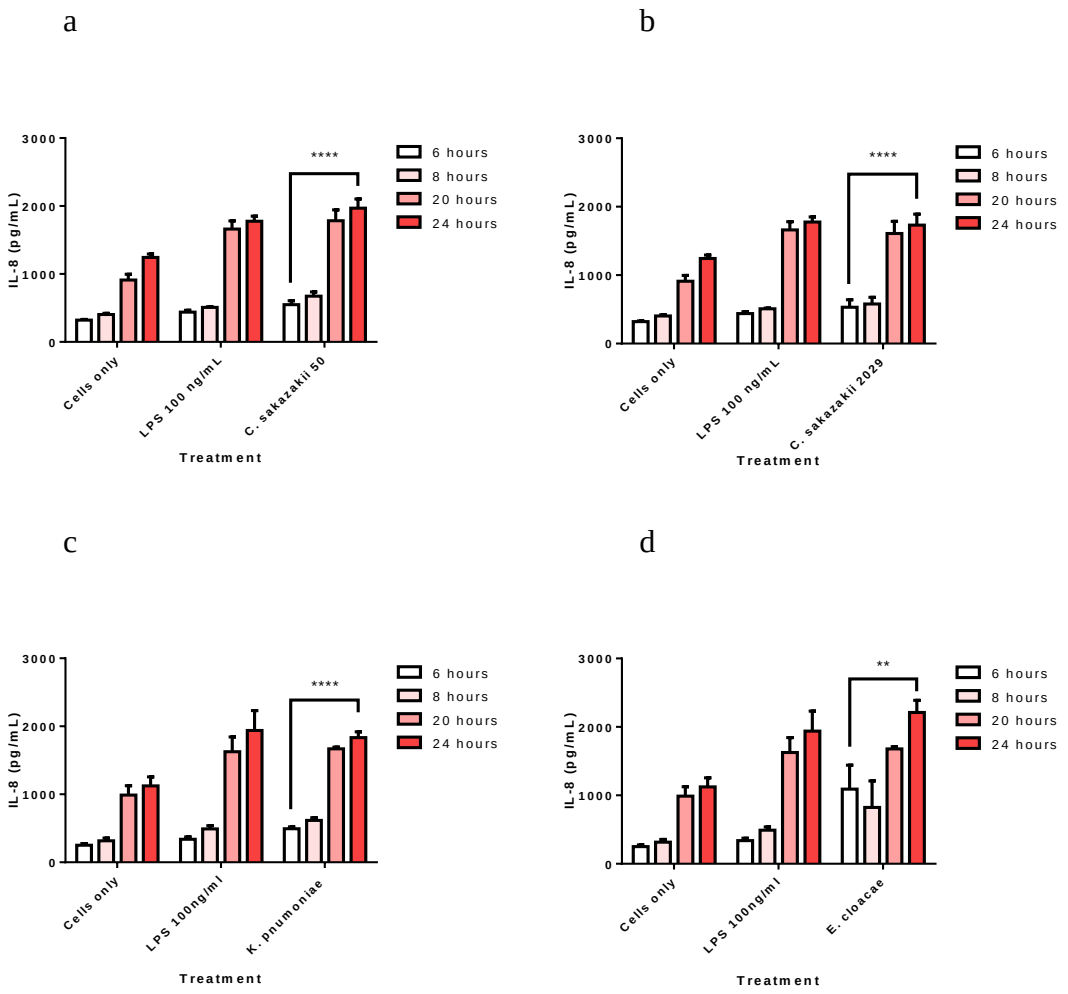
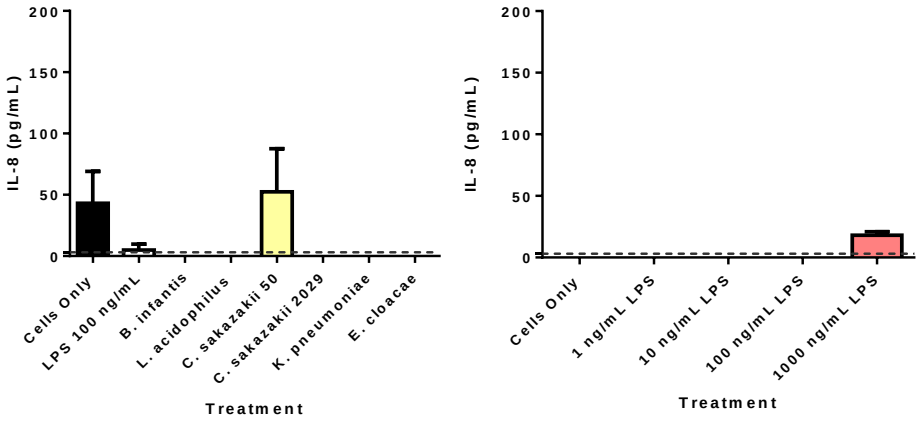
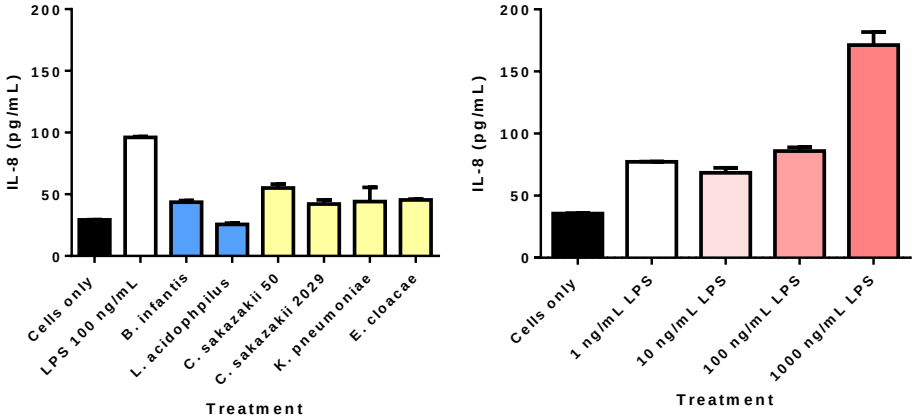


Figure 3.4. IL-8 production by HT-29 cells over time in response to four strains of heat inactivated NEC-associated bacteria. Semi-confluent HT-29 cells were incubated for 24 h with $1x10^7$ cfu/mL of washed and heat-killed bacteria and samples were taken at 6, 8, 20 and 24 h post stimulation. Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Statistical analysis; two way ANOVA with multiple comparisons (Tukey's multiple comparison test). ** $p < 0.01$ and **** $p < 0.0001$. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

a Undifferentiated Caco-2 cells



b Partially differentiated Caco-2 cells



c Completely differentiated Caco-2 cells

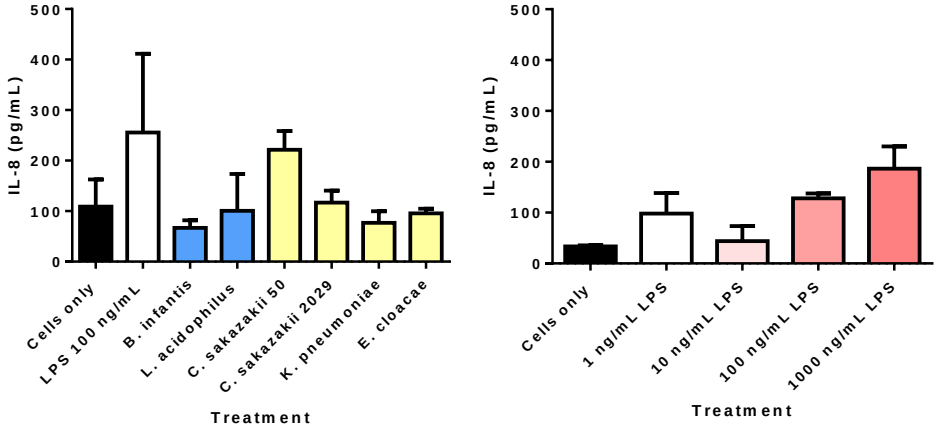


Figure 3.5. IL-8 production by Caco-2 cells in response to LPS and heat inactivated NEC-associated bacteria. Non-differentiated (a), partially differentiated (b) and completely differentiated (c) Caco-2 cells were incubated for 24 h with $1x10^7$ cfu/mL washed, heat-killed *B. infantis, L. acidophilus, C. sakazakii* 50, *C. sakazakii* 2029, *K. pneumoniae* VIII 8 or *E. cloacae* I 1 (left column) or various concentrations of LPS (right column). Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Bars and error bars represent the mean and the SEM of duplicate wells of one experiment. Dotted grey horizontal line (a) represents ELISA detection limit: 3.1 pg/mL.

Since it was determined that four NEC-associated bacteria could induce an IL-8 response it was next decided to see if bacteria isolated from control, NEC suspected or NEC confirmed infant groups from the NICU would elicit a response. Prior to the stimulation assays, doubling dilutions and optical densities for each organism were conducted (Appendix 5.2, Figure 5.2). All bacteria reached similar concentrations, achieving an OD of approximately 1 and a concentration of between $1.5x10^9$ and $2\ x10^9$ cfu/mL after 24 h. Growth curves and doubling dilutions allowed for accurate estimation of bacterial numbers during subsequent assays. In the stimulation assay, no significant difference in IL-8 production compared to the cells only control was found after 24 h in response to any bacterial isolate at $1x10^6$ cfu/mL (Figure 3.6 a). Approximately half of the bacterial isolates induced a significant ($p < .05$) IL-8 response compared to the cells only controls by HT-29 cells at $1x10^7$ cfu/mL (Figure 3.6 b). All bacterial isolates elicited a strong and significant ($p < 0.0001$) response at $1x10^8$ cfu/mL (Figure 3.6 c). There was no significant difference between the IL-8 production by HT-29 cells in response to the different clinically isolated bacteria at $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL, regardless of the NEC group they were isolated from (Appendix 5.2, Figure 5.11). Taken together, this data indicates that *Enterobacteriaceae* isolated from infants in the NICU were able to elicit an IL-8 response from HT-29 cells but bacteria from different NEC infants groups did not differ in their ability to induce a response.

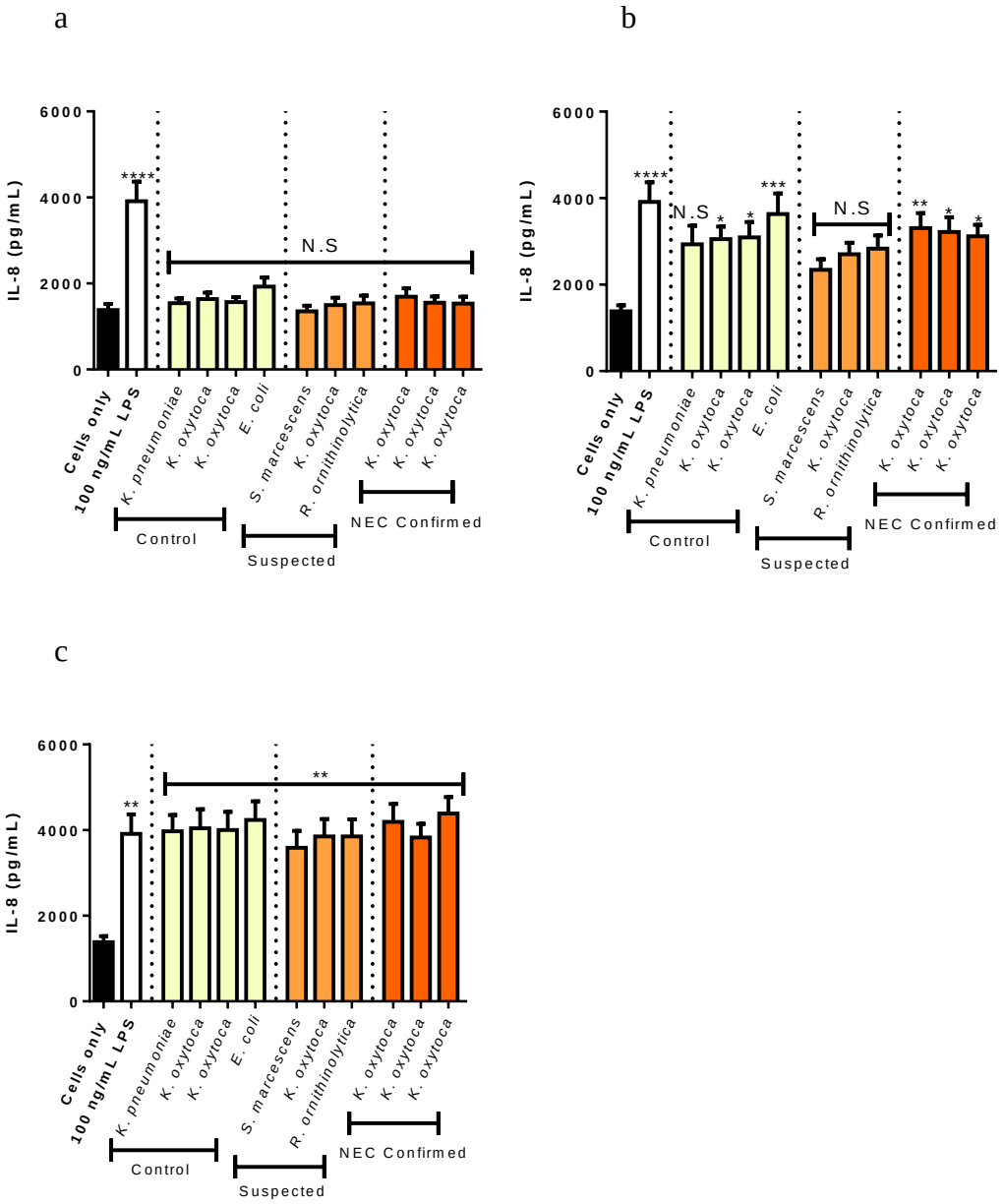


Figure 3.6. IL-8 production by HT-29 cells in response to clinically isolated bacteria from different NEC group infants. Semi-confluent HT-29 cells were incubated with $1x10^6$ (a) $1x10^7$ (b) or $1x10^8$ cfu/mL (c) of 10 different strains of washed, heat-killed *Enterobacteriaceae* for 24 h. Bacteria were originally isolated from control, suspected or NEC confirmed infants (Table 2.2). Media only and 100 ng/mL LPS were used as the negative and positive controls. Statistical analysis; one way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, compared to the cells only control. Bars and error bars represent the pooled mean and the SEM of duplicate wells of four separate experiments.

3.2 Probiotic immunomodulation

3.2.1 Probiotic bacterial enumeration

Because the NEC-associated coliform bacteria were able to induce an IL-8 response in HT-29 cells, it was hypothesised that the probiotic bacteria *B. infantis* or *L. acidophilus*, used clinically to prevent NEC may not elicit an IL-8 response. To ensure estimation of bacterial counts for future experiments, colony forming units were calculated using the method described in 2.6 (Figure 3.7). *B. infantis* reached $7x10^8$ cfu/mL and an OD of 1.6 while *L. acidophilus* reached $8x10^8$ cfu/mL at an OD of 1.7.

a b

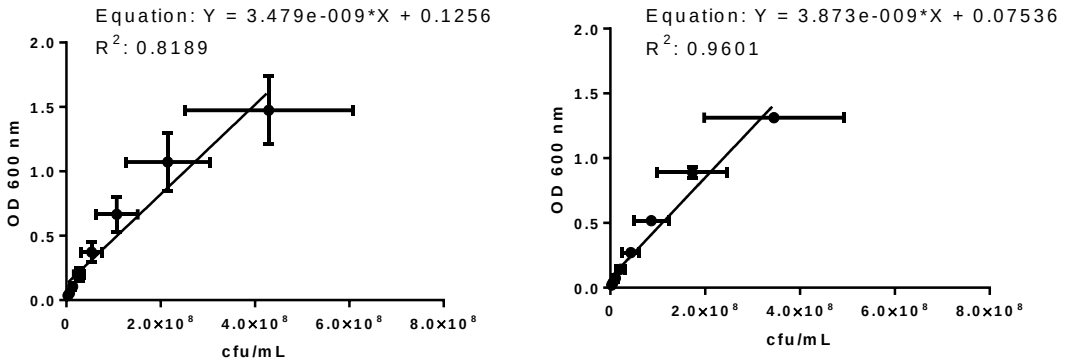


Figure 3.7. Estimation of probiotic bacterial numbers. The probiotic bacteria *B. infantis* (a) and *L. acidophilus* (b) were incubated for 48 h, 37°C in MRS broths. ODs and cfu/mL were determined as indicated in the methods (Section 2.2). OD was plotted against cfu/mL. Data points and error bars represent the pooled mean and the SEM of three separate experiments. Statistical analysis; linear regression. Eight data points are shown in each curve.

3.2.2 IL-8 production by HT-29 cells in response to heat-killed probiotic bacteria

The heat-killed probiotic bacteria from Infloran® were incubated with HT-29 cells at $1x10^6$, $1x10^7$ and $1x10^8$ cfu/mL (Figure 3.8 a and b). However, unlike the NEC-associated coliform bacteria (Figure 3.3-3.6), the probiotic bacteria did not induce any significant change in IL-8 production compared to the cells only controls after 24 h, regardless of concentration. It was also found that Caco-2 cells did not respond to heat-killed probiotics by producing IL-8 (Figure 3.5 b and c).The constitutive production in IL-8 by HT-29 cells remained the same throughout the experiment. This data shows that the probiotic bacteria from Infloran® do not induce a pro-inflammatory response as measured by IL-8 production from enterocytes *in vitro*.

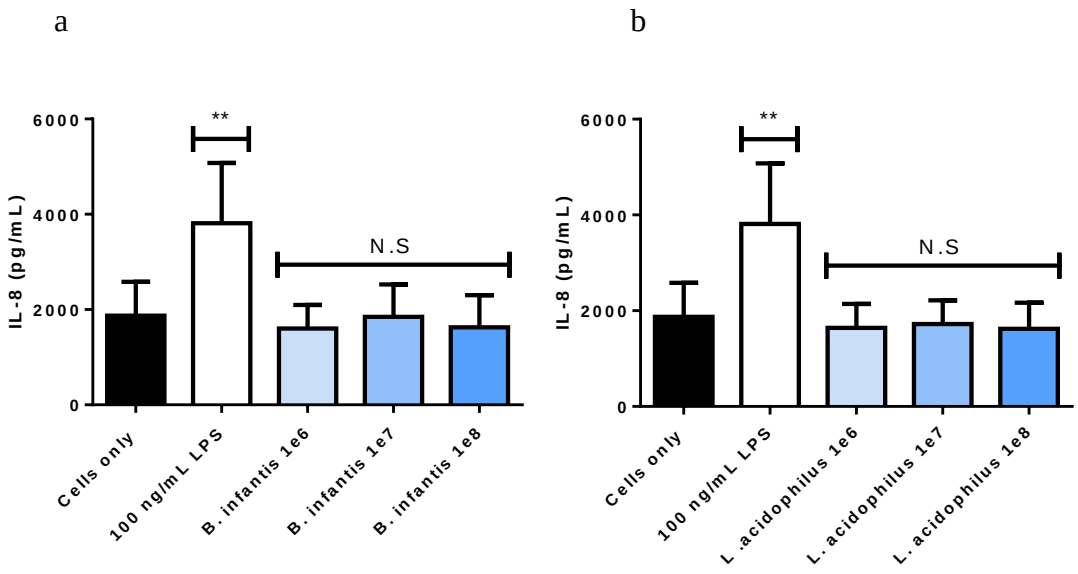


Figure 3.8. IL-8 production by HT-29 cells in response to heat-killed probiotic bacteria. Semi-confluent HT-29 cells were incubated for 24 h with $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL of washed, heat-killed *B. infantis* (a) or *L. acidophilus* (b). Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Statistical analysis; one way ANOVA with multiple comparisons (Dunnet's multiple comparison test), N.S, not significant; ** $p < 0.01$ compared to the cells only control. Bars and error bars represent the pooled mean and the SEM of duplicate wells of three separate experiments.

3.2.3 Effect of heat-killed probiotics on LPS induced IL-8 production

The probiotic bacteria *B. infantis* and *L. acidophilus* are used clinically to prevent NEC and are postulated to have a number of different effects on the host, including immunomodulation. It was decided to see if they could reduce the IL-8 production in response to LPS or NEC-associated bacteria.

Firstly, to see if the probiotics could reduce the response seen against a bacterial endotoxin, HT-29 cells were co-incubated with $1x10^7$ cfu/mL heat-killed *B. infantis* or *L. acidophilu*s and 100 ng/mL LPS. However, no reduction in IL-8 production was found (Appendix 5.2, Figure 5.3). Secondly, we hypothesised that the probiotics may need to be incubated with the HT-29 cells prior to the LPS stimulation, a technique which is commonly seen in probiotic immunomodulatory assays[145]. However, it was found that a 1, 3 or 6 h prime with the two probiotic strains was insufficient to reduce the IL-8 produced (Appendix, Figure 5.4).

Next, it was thought that the signal generated by purified LPS may be too strong for the probiotics to reduce the response seen. Therefore, HT-29 cells were pre-treated with probiotic bacteria before adding the four NEC-associated bacteria to determine if IL-8 production induced by the bacteria could be reduced. However, it was seen that after a 6 h (Figure 3.9) or 24 hour (Appendix 5.2, Figure 5.5) prime with $1x10^7$ cfu/mL heat-killed *B. infantis* or *L. acidophilus* and subsequent stimulation with the different *Enterobacteriaceae*, the IL-8 produced was not significantly different from that produced by the NEC-associated bacteria alone. IL-8 production is expressed as fold changed compared to the cells only controls in this case, as the cells only controls exhibited large variation during these experiments, possibly due to the cells being at different stages of the cell cycle, or slightly different cell numbers. Different concentrations of probiotic bacteria were also tried (Appendix 5.2, Figure 5.6). Taken together this data shows that although heat-killed probiotic bacteria do not induce an IL-8 response by IECs, they are unable to reduce it in response to *Enterobacteriaceae* or LPS.

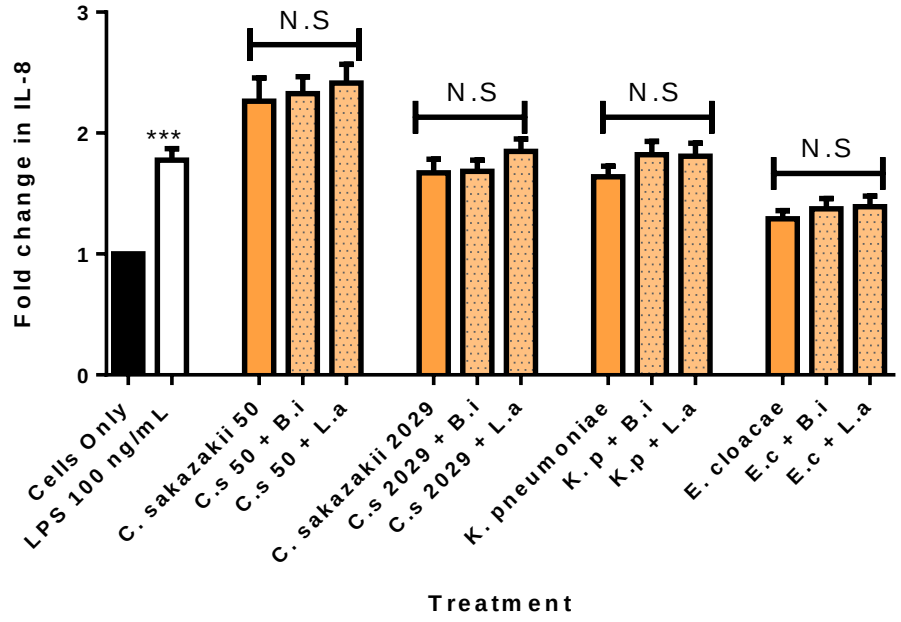


Figure 3.9. IL-8 production by HT-29 cells in response to NEC-associated bacteria after a probiotic prime. Semi-confluent HT-29 cells were incubated for 6 h with $1x10^7$ cfu/mL of heat-killed, washed probiotic bacteria prior to the addition and 24 h incubation with $1x10^7$ cfu/mL heat-killed, washed NEC-associated bacteria. Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Statistical analysis; one way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S, no significant difference between treatments within this group, and *** $p < 0.001$ compared to cells only control. Bars and error bars represent the pooled mean and the SEM of duplicate wells of six separate experiments. Values depicted are expressed as fold change in IL-8 production compared to the cells only control in each replicate experiment. + B.i indicates a prime with *B. infantis* and + L.a indicates a prime with *L. acidophilus*.

3.2.4 Effect of bacterial lipoteichoic acid on IL-8 production

It has previously been shown that the Gram positive cell wall component and TLR2 agonist, lipoteichoic acid is able reduce pro-inflammatory responses[151]. Therefore, the IL-8 production in response to LPS in the presence of LTA by HT-29 cells was investigated. HT-29 cells were incubated with various concentrations of LTA alone or with 100 ng/mL LPS for 24 h and the IL-8 production was measured by ELISA. The highest concentration of LTA (100 µg/mL) resulted in a significant ($p < 0.0001$) reduction in IL-8 production in response to LPS (Figure 3.10), while the lower concentrations did not affect the IL-8 production. There was no significant difference between the cells only control and the LTA only treatments (Appendix, Figure 5.13).

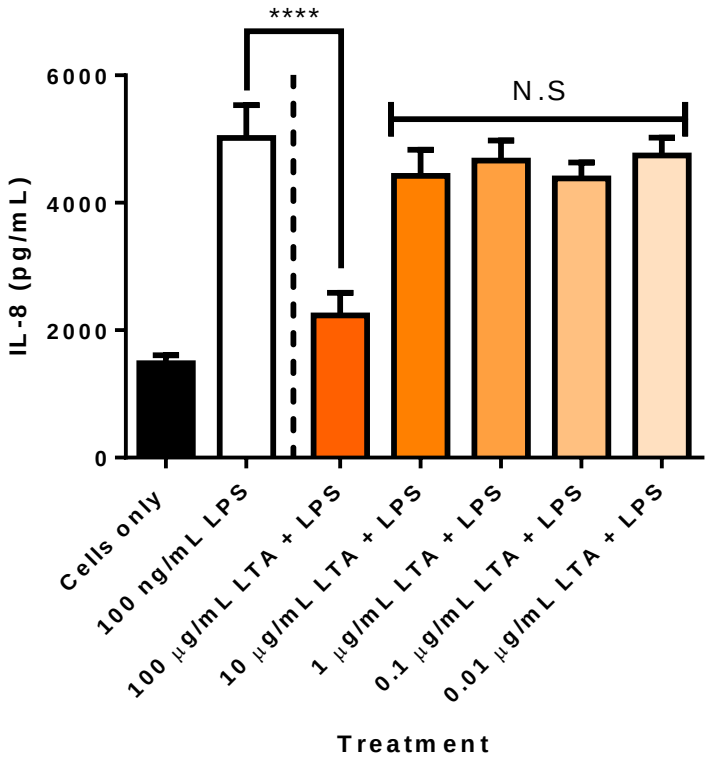


Figure 3.10. IL-8 production by HT-29 cells in response to LPS and LTA. Semi-confluent HT-29 cells were incubated for 24 h with various concentrations of LTA with or without 100 ng/mL of *E. coli* LPS. Statistical analysis; ordinary two way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S, not significant compared to LPS stimulation alone and **** $p < 0.0001$. Bars and error bars represent the pooled mean and the SEM of duplicate wells of three separate experiments.

3.2.5 IL-8 production by HT-29 cells in the presence of live Infloran® bacteria

Since heat-killed probiotic bacteria were unable to reduce the IL-8 production by HT-29 cells, it was next decided to see if live probiotics could reduce IL-8 production. Live or heat-killed *B. infantis* or *L. acidophilus* were incubated with HT-29 cells alone or with 100 ng/mL LPS. Different concentrations of bacteria were included to ensure enough bacteria were present in order to have an effect. It was found that, as before (Appendix 5.2, Figure 5.3 c), heat-killed probiotic bacteria caused no significant reduction in IL-8 production, regardless of the concentration of probiotic used (Figure 3.11 a and b). Also, in response to live *L. acidophilus* the IL-8 production was not affected (Figure 3.11 b). However, the IL-8 production in response to LPS was significantly decreased with a co-incubation of live $1x10^6$ ($p < 0.001$), $1x10^7$ ($p < 0.0001$) or $1x10^8$ cfu/mL *B. infantis* compared to the LPS stimulation ($p < 0.0001$, Figure 3.11 a).

The viability of the HT-29 cells was determined after the co-incubation with live probiotic bacteria in order to check if the significant reduction in IL-8 production was associated with a decrease in HT-29 cell viability (same as highlighted in section 2.8). The highest concentration of *B. infantis* ($1x10^8$ cfu/mL) decreased the viability of the HT-29 cells to 58% (in comparison, the cells only and LPS control viabilities were approximately 93% and 98% respectively, while the highest concentration of live *L. acidophilus* resulted in an HT-29 cell viability of 92%). The viability of the HT-29 cells stimulated with fewer *B. infantis* ($1x10^7$ and $1x10^6$ cfu/mL) were not drastically changed (both 89%). The viability of the HT-29 cells incubated with the highest concentration of heat-killed *B. infantis* or *L. acidophilus* ($1x10^8$ cfu/mL) was not affected (96% and 95% respectively). It must be noted that in this case the viability assays were only carried out once (due to time constraints), and therefore caution should be used when interpreting this data. During similar studies where differences in IL-8 production were seen, some authors commented on IEC viability[145,157,158], while others did not[144,151,155,159].

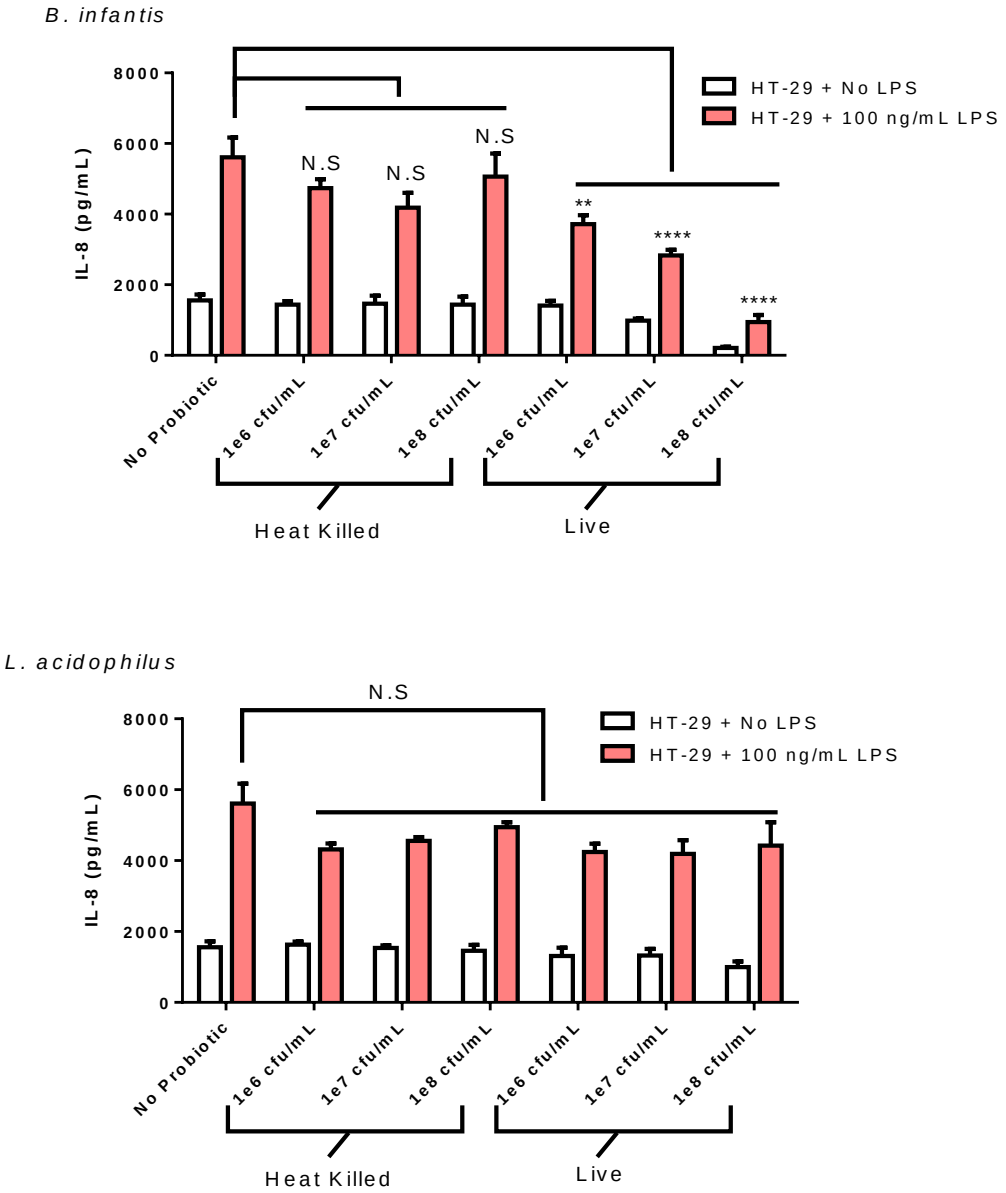


Figure 3.11. IL-8 production by HT-29 cells in response to LPS and live probiotic bacteria. Semi-confluent HT-29 cells were incubated for 24 h with or without $1x10^6$, $1x10^7$ or $1x10^8$ cfu/mL live or heat-killed *B. infantis* (top) or *L. acidophilus* (bottom) with or without 100 ng/mL LPS. Media only and 100 ng/mL LPS without bacteria were used as the negative and positive controls respectively. Statistical analysis; ordinary two way ANOVA with multiple comparisons (Sidak's multiple comparisons test). N.S, not significant, ** $p < 0.01$ and **** $p < 0.0001$. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

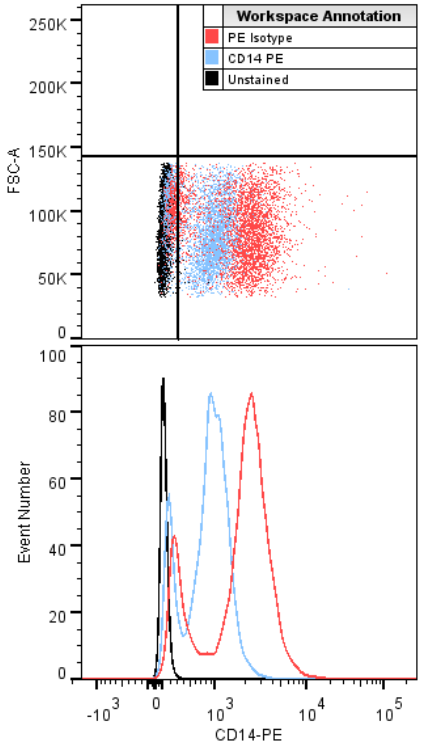
3.3 CD14 and TLR4 expression by HT-29 cells

The analysis of surface markers on HT-29 cells using flow cytometry had not been undertaken by this laboratory group before. Therefore, considerable optimisation had to be undertaken in order to determine the CD14 and TLR4 expression using this method. A number of techniques and protocols were incorporated in order to accurately measure marker expression, and are described in the following sections.

3.3.1 CD14 expression on HT-29 cells measured by flow cytometry

CD14 is the co-receptor for LPS and, alongside TLR4, can contribute to the cell's ability to recognise bacterial endotoxin. To determine the role of CD14 in LPS signalling in enterocytes, the CD14 expression was measured on HT-29 cells by flow cytometry. Early experiments indicated that non-specific antibody binding may have occurred, as indicated by the isotype control (Figure 3.12 a and b).

a



b

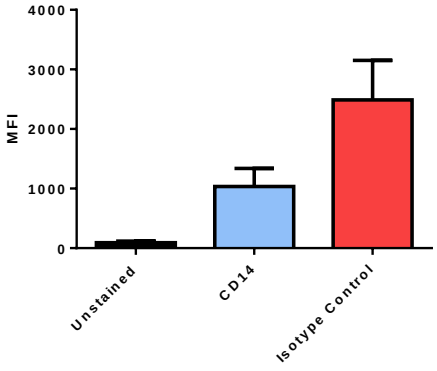


Figure 3.12. CD14 expression on HT-29 cells measured by flow cytometry. Semi-confluent HT-29 cells were incubated for 24 h with no stimulus and stained with an anti-CD14 or isotype control antibody or left unstained. Dot-plot (a, top) and histogram (a, bottom) are representative plots of a typical result. Graph (b) depicts the median fluorescence intensity (MFI) of the entire live HT-29 cell population. Bars and error bars represent the pooled mean and the SEM of four separate experiments. Ten thousand events were recorded for each experiment. Quad gate indicates the PE negative population gated on the unstained control. FSC-A; forward scatter area.

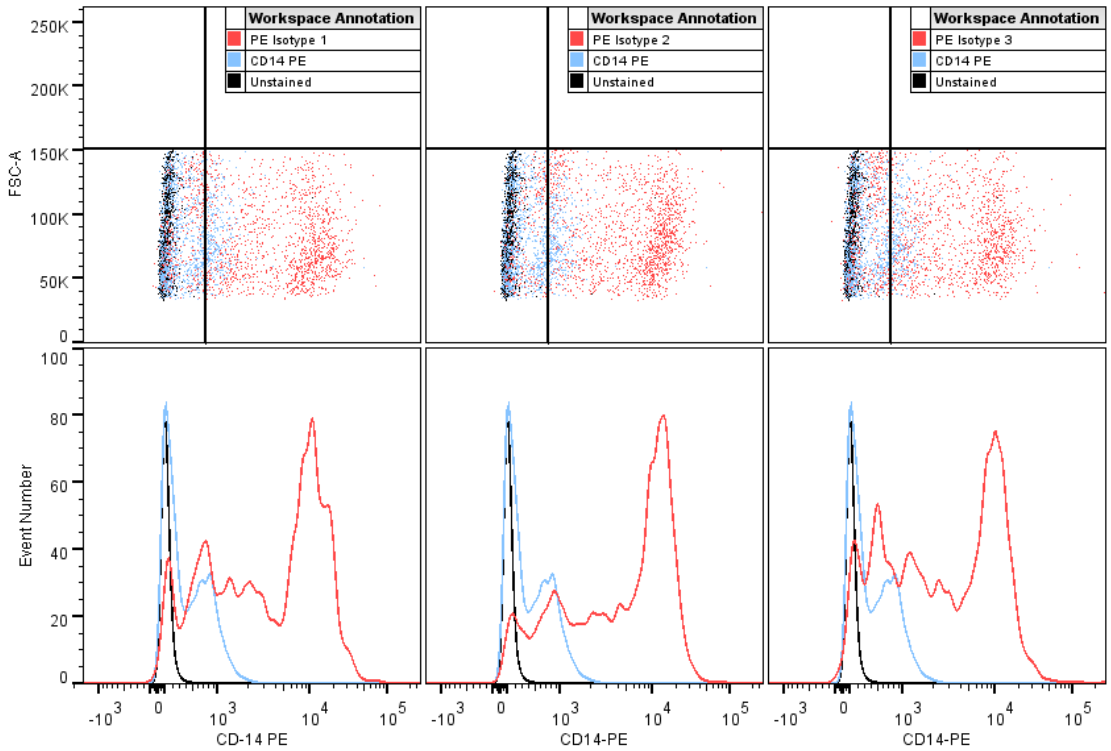
3.3.2 Optimisation of non-specific antibody binding

Isotype control antibodies can produce erroneous results[160] so different isotype controls were tried. However two different isotype control antibodies also resulted in a large amount of non-specific antibody binding (Figure 3.13 a). Therefore, it was decided to incorporate an FcR block before antibody staining. However, non-specific binding still occurred as indicated by the isotype control (Figure 3.13 b).

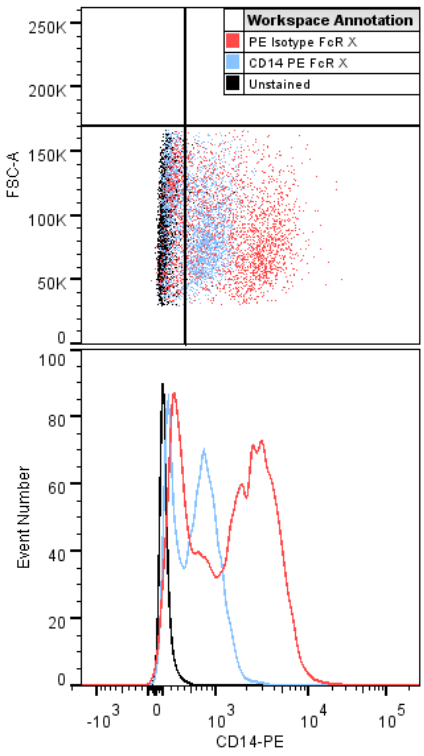
Other techniques used in an effort to reduce the non-specific antibody binding issue included: Antibody titrations, introduction of live/dead stains and dissociation of cells using different methods and reagents, none of which resulted in decreased nonspecific antibody binding (highlighted in Appendix, Table 5.1).

Different CD14 and isotype control antibodies, conjugated to brilliant violet instead of PE, resulted in the complete ablation of non-specific antibody binding (Figure 3.13 c).

a


Workspace Annotation
PE Isotype 1
CD14 PE
Unstained
Workspace Annotation
PE Isotype 2
CD14 PE
Unstained
Workspace Annotation
PE Isotype 3
CD14 PE
Unstained
FSC-A
Event Number
CD-14 PE
CD14-PE
CD14-PE

b


Workspace Annotation
PE Isotype FcR X
CD14 PE FcR X
Unstained
FSC-A
Event Number
CD14-PE

c

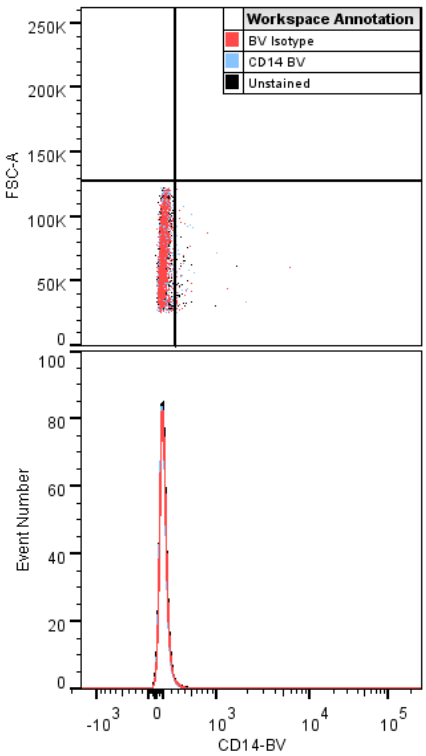

Workspace Annotation
BV Isotype
CD14 BV
Unstained
FSC-A
Event Number
CD14-BV

Figure 3.13. Optimisation of non-specific antibody binding. Semi-confluent HT-29 cells were incubated for 24 h with no stimulus and then stained with anti-CD14 or one of three different isotype control antibodies (a). Isotype 1: PE mouse IgG2a-κ isotype control (eBM2a, eBioscience), isotype 2: PE rat IgG2a-κ isotype control (eBR2a, eBioscience) or isotype 3: PE mouse IgG-κ isotype control (MOPC-21, BioLegend). Conversely, HT-29 cells were incubated for 24 h with no stimulus and blocked with an FcR block (FcR X) prior to antibody staining (b). Alternatively, HT-29 cells were incubated for 24 h with no stimulus and then stained with a CD14 specific antibody conjugated to brilliant violet or a brilliant violet isotype control antibody(c) or left unstained. Ten thousand events were recorded in each independent experiment.

3.3.3 CD14 positive control

A CD14 positive control was then introduced to ensure that CD14 could be reliably detected. THP-1 cells have been shown to express CD14[161] and have previously been used as a CD14 positive control during flow cytometry experiments[162,163]. Therefore, we investigated the amount of CD14 expressed by undifferentiated or differentiated THP-1 cells. However, we were unable to detect expression of CD14 on either differentiated or undifferentiated THP-1 cells (Figure 3.14 a). PBMCs are also known to express CD14[164] and are often used as a CD14 positive control[119]. Therefore, CD14 expression by PBMCs was investigated. PBMCs exhibited high levels of CD14 expression (Figure 3.14 b) and, as a result PBMCs were used as the CD14 positive control. As PBMCs showed sufficient CD14 expression, THP-1 cells were not further investigated as a positive control. Brilliant violet antibody titration was carried out on PBMCs (Appendix 5.2, Figure 5.8).

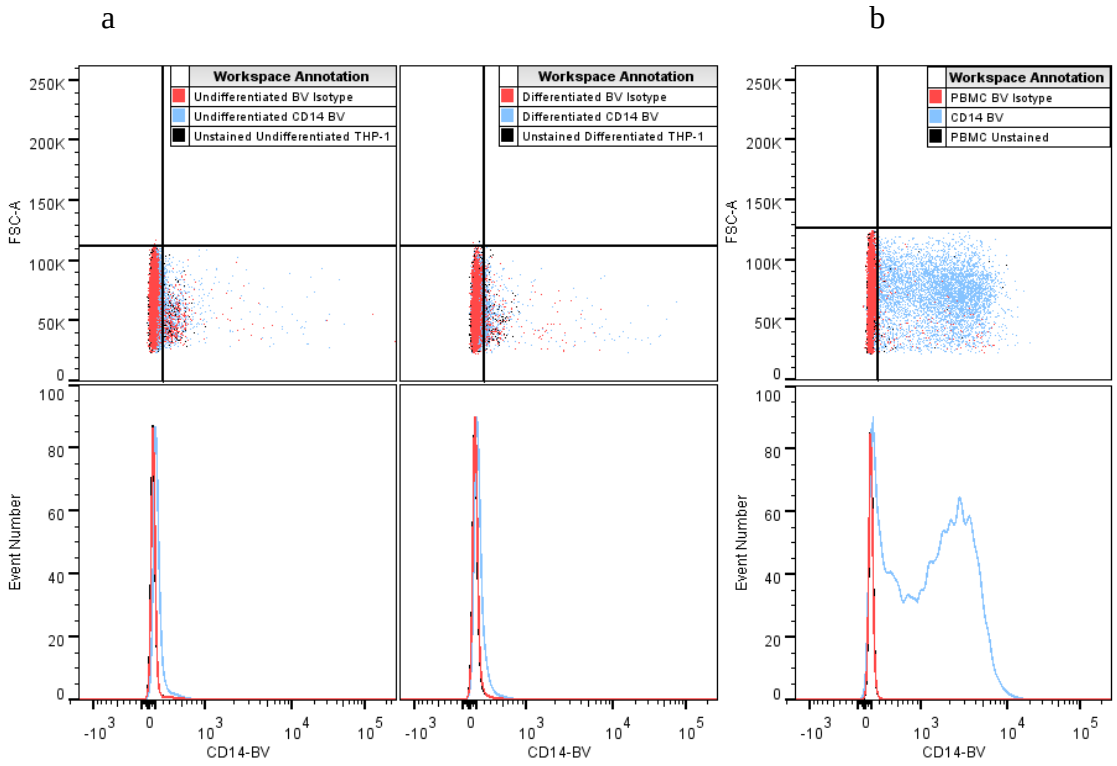


Figure 3.14. PBMCs as a CD14 positive control. Semi-confluent THP-1 cells were incubated for 24 h with no stimulus (left column) or with 5 ng/mL 2-ME (right column) and then stained with anti-CD14, an isotype control antibody or left unstained (a). Alternatively, PBMCs were cultured from human blood with no stimulus and stained with anti-CD14, an isotype control antibody or left unstained (b). Ten thousand events were recorded for each experiment.

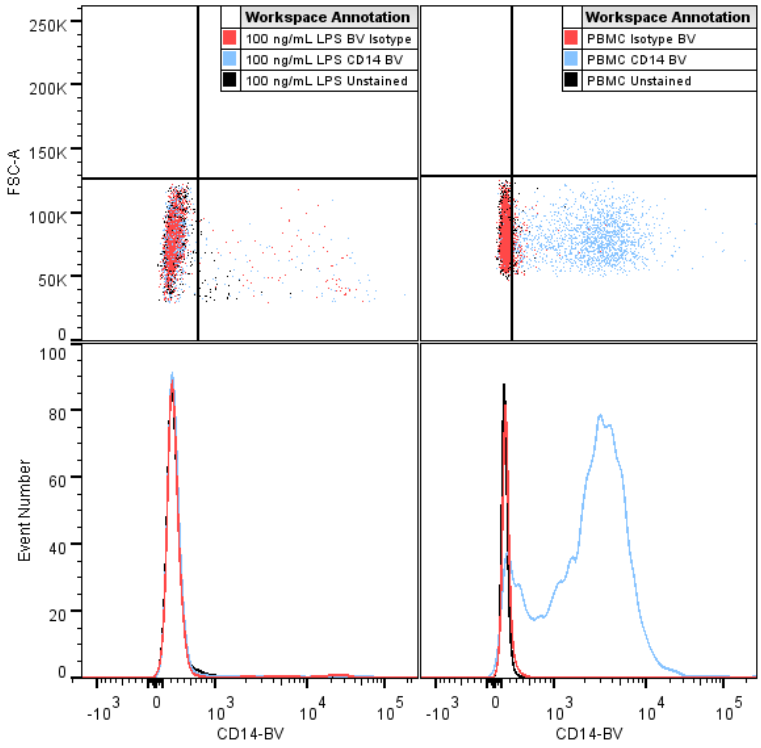
3.3.4 CD14 expression on HT-29 cells measured by flow cytometry

In certain cells, it has been shown that CD14 expression levels can change with an LPS stimulation[120,125]. Therefore, the CD14 expression by HT-29 cells was tested in response to different concentrations of LPS (Figure 3.15). CD14 was undetectable on HT-29 cells regardless of LPS concentration (Figure 3.15 b), despite the PBMC positive control showing that CD14 was detectable under these experimental conditions.

Intracellular staining of HT-29 cells was also carried out to detect soluble CD14 before its release (data not shown). However, a large amount of isotype staining meant no reliable data was obtained.

CD14 expression on unstimulated (Figure 3.16) and stimulated (Appendix, Figure 5.12) Caco-2 cells (known to express CD14[119,125]) was also tested by flow cytometry. However, CD14 was undetectable.

a



b

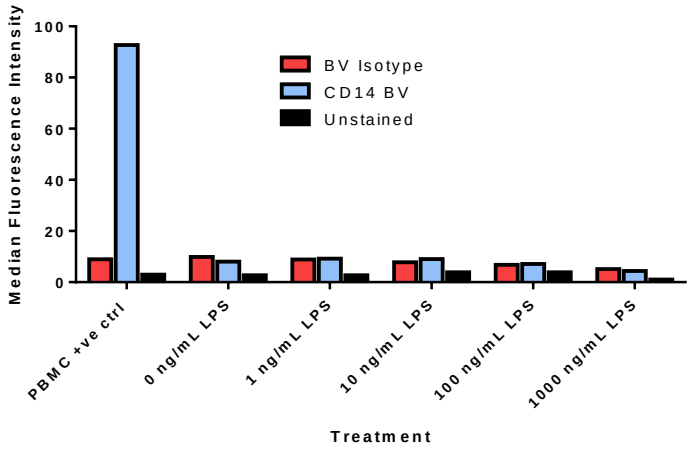


Figure 3.15. CD14 expression on HT-29 cells by flow cytometry. Semi-confluent HT-29 cells were cultured for 24 hours with 0-1000 ng/mL LPS for 24 hours then stained with an anti-CD14 or isotype control antibody or left unstained (left column). PBMCs were cultured with no stimulus and stained as for the HT-29 cells (right column) (a). Graph (b) depicts the MFI of the entire live HT-29 cell population. Ten thousand events were recorded for each experiment.

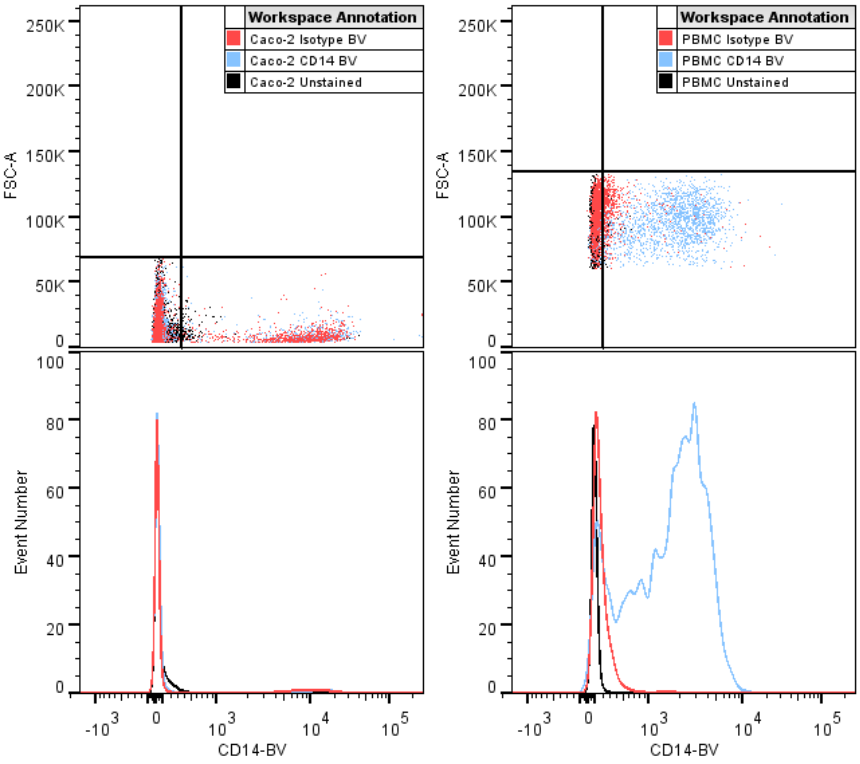
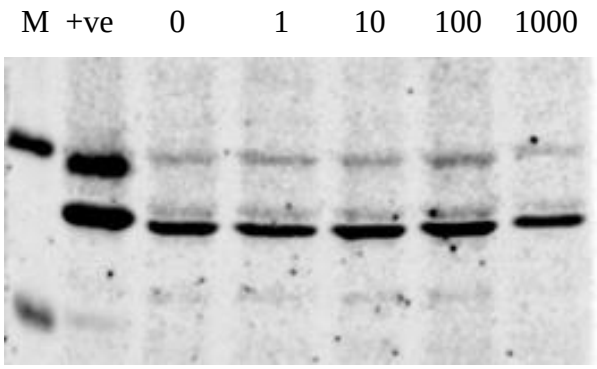


Figure 3.16. CD14 expression on Caco-2 cells by flow cytometry. Semi-confluent Caco-2 cells were cultured unstimulated for 24 hours and stained with an anti-CD14 or isotype control antibody or left unstained (left column). PBMCs were cultured with no stimulus and stained as for the Caco-2 cells as a positive control (right column). Ten thousand events were recorded.

3.3.5 CD14 expression on HT-29 cells measured by Western blotting

As CD14 expression on enterocytes was not detected by flow cytometry, it was decided to determine if the LPS co-receptor could be detected using Western blotting. CD14 protein was detected from PBMCs and also from HT-29 cells (Figure 3.17 a). HT-29 cells showed the presence of two different forms of CD14, indicated by two different size bands (approximately 54 and 48 kDa). These bands correspond to the correct size of different CD14 molecules (56, 54 or 48 kDa depending on type). CD14 expression by HT-29 cells in response to LPS was the same regardless of LPS concentration (Figure 3.17 b and c).

a


M
+ve
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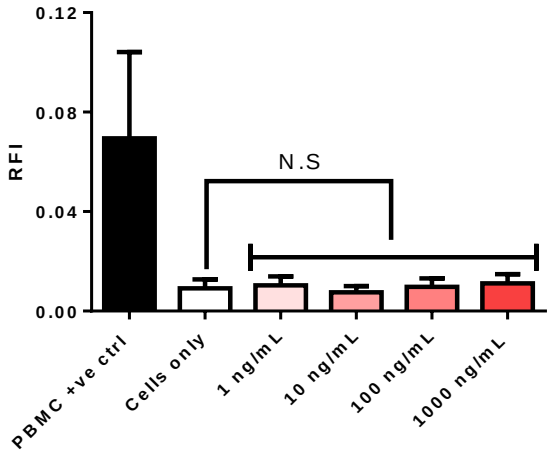
b


0.12
0.08
0.04
0.00
RFI
N.S
PBMC +ve ctrl
Cells only
1 ng/mL
10 ng/mL
100 ng/mL
1000 ng/mL

c

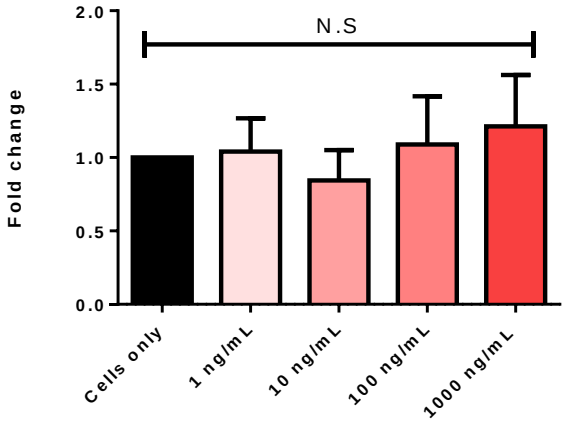

2.0
1.5
1.0
0.5
0.0
Fold change
N.S
Cells only
1 ng/mL
10 ng/mL
100 ng/mL
1000 ng/mL

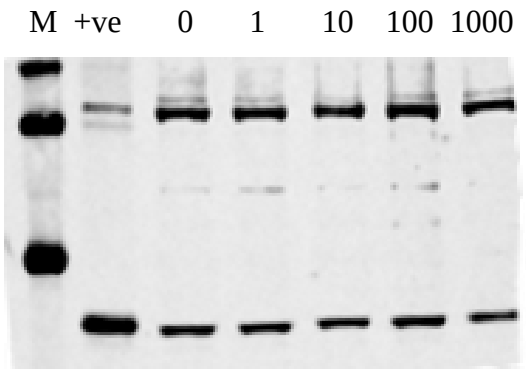
Figure 3.17. HT-29 cells express CD14 as measured by Western blotting. Western blot image showing the presence of CD14 (a), the relative fluorescence intensity (RFI) of CD14 signal when normalised to actin (b) and the fold change of RFI compared to the cells only controls (c). M; molecular weight marker, +ve; PBMC CD14 positive control, 0-1000; LPS concentration in ng/mL HT-29 cells were stimulated with during the assay. Actin served as a loading control. Molecular weight marker indicates approximately 55 kDa (highest band) and 35 kDa (lowest band). Fluorescence was quantified using image studio software. Statistical analysis; one way ANOVA with multiple comparisons (Dunnett's multiple comparisons test). N.S, no significant difference. Bars and error bars represent the pooled mean and the SEM of four separate experiments.

3.3.6 TLR4 expression on HT-29 cells measured by Western blotting

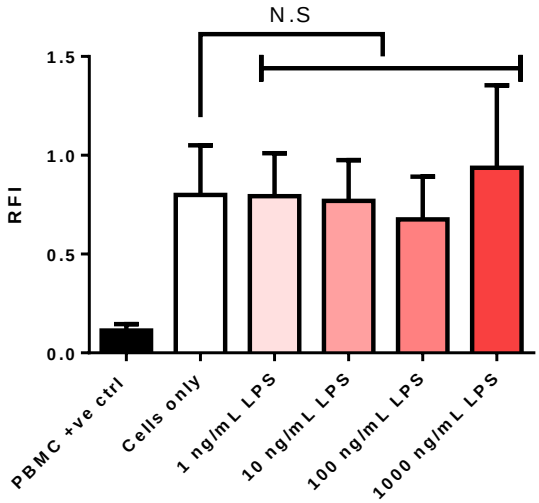
TLR4, the putative endotoxin receptor, is involved in the detection of LPS alongside CD14. The expression of TLR4 by HT-29 cells was examined by Western blot (Figure 3.18). A band was detected at approximately 100 kDa, close to the expected size of TLR4 (95 kDa), in the PBMC positive control and also in all treatments of the HT-29 cells. Although HT-29 cells express TLR4, the expression levels did not differ with various LPS concentrations (Figure 3.18 b and c). Replicates of the CD14 and TLR4 Western blots can be found in the appendix (Appendix 5.2, Figure 5.9 and 5.10).

Taken together, this data shows that HT-29 cells express the LPS receptor, TLR4 and also the LPS co-receptor CD14, however LPS stimulation at the concentrations used did not affect their relative expression.

a


M
+ve
0
1
10
100
1000

b


N.S
RFI
1.5
1.0
0.5
0.0
PBMC +ve ctrl
Cells only
1 ng/mL LPS
10 ng/mL LPS
100 ng/mL LPS
1000 ng/mL LPS

c

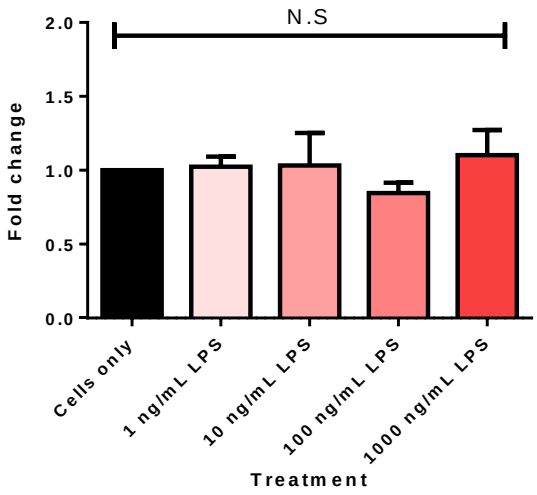

N.S
Fold change
2.0
1.5
1.0
0.5
0.0
Cells only
1 ng/mL LPS
10 ng/mL LPS
100 ng/mL LPS
1000 ng/mL LPS
Treatment

Figure 3.18. HT-29 cells express TLR4 as measured by Western blotting. Western blot image showing the presence of TLR4 (a), the RFI of TLR4 signal when normalised to actin (b) and the fold change of RFI compared to the cells only control (c). M; molecular weight marker, +ve; PBMC TLR4 positive control, 0-1000; LPS concentration in ng/mL with which the HT-29 cells were stimulated with, during the assay. Actin served as a loading control. Molecular weight marker indicates approximately 130 kDa (top band), 100 kDa (middle band) and 55 kDa (bottom band). Fluorescence was quantified using image studio software. Statistical analysis; one way ANOVA with multiple comparisons (Dunnett's multiple comparisons test). N.S, no significant difference. Bars and error bars represent the pooled mean and the SEM of four separate experiments.

3.3.7 The role of CD14 in the LPS-induced IL-8 production by HT-29 cells

As both TLR4 and CD14 were detected on HT-29 cells, the importance of these receptors for LPS signalling was next investigated. HT-29 cells were incubated with an anti-CD14 antibody before (Figure 3.19 a and b) or during (Figure 3.19 c) the LPS stimulation and the IL-8 production was measured. The highest concentration of blocking antibody (5 µg/mL) significantly reduced ($p < 0.05$) the IL-8 production by HT-29 cells during the 1 hour pre-incubation (Figure 3.19 a). Although the 3 hour pre-incubation reduced the IL-8 production (Figure 3.19 b), this was not significant. IL-8 production was significantly reduced ($p < 0.01$) to levels resembling the unstimulated control with a number of different blocking antibody concentrations (1.25-0.31 µg/mL) when incubated with LPS over a 24 h period (Figure 3.19 c). Due to limiting amounts of blocking antibody, half the original concentration of blocking antibody and LPS were used during this experiment compared to the pre-incubation experiment. Taken together, this data suggests that LPS induced IL-8 production by HT-29 cells is, at least in part, dependent on CD14.

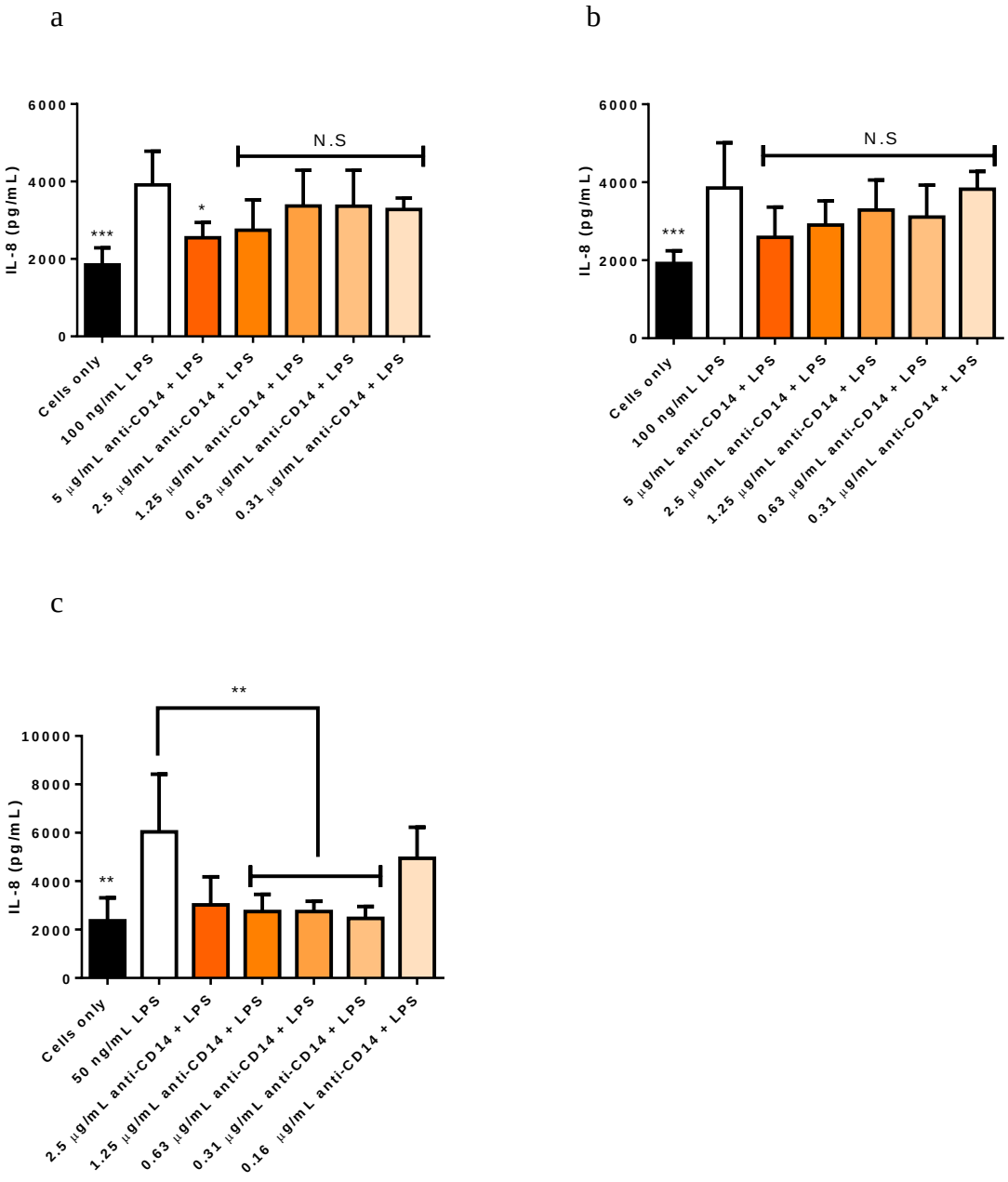


Figure 3.19. IL-8 production in response to anti-CD14 and LPS. Semi-confluent HT-29 cells were incubated for 1 h (a) or 3 h (b) with 5-0.31 µg/mL anti-CD14 prior to a 24 h stimulation with 100 ng/mL *E. coli* LPS. Alternatively, HT-29 cells were incubated for 24 h with 2.5-0.16 µg/mL anti-CD14 during a stimulation with 50 ng/mL *E. coli* LPS (c). Statistical analysis; one way ANOVA with multiple comparisons (Dunnet's multiple comparison test). N.S, not significant; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ compared to LPS stimulation alone. Bars and error bars represent the pooled mean and the SEM of duplicate wells of three separate experiments.

3.3.8 The role of TLR4 in the LPS induced IL-8 production by HT-29 cells

Since it was demonstrated that CD14 was required for LPS induced HT-29 cell IL-8 production, we investigated whether TLR4 was also required for LPS induced IL-8 production by HT-29 cells. However, it was found that at the concentrations of anti-TLR4 antibody used, the LPS induced IL-8 production could not be reduced (Figure 3.20), regardless of whether a prime or co-incubation was utilised.

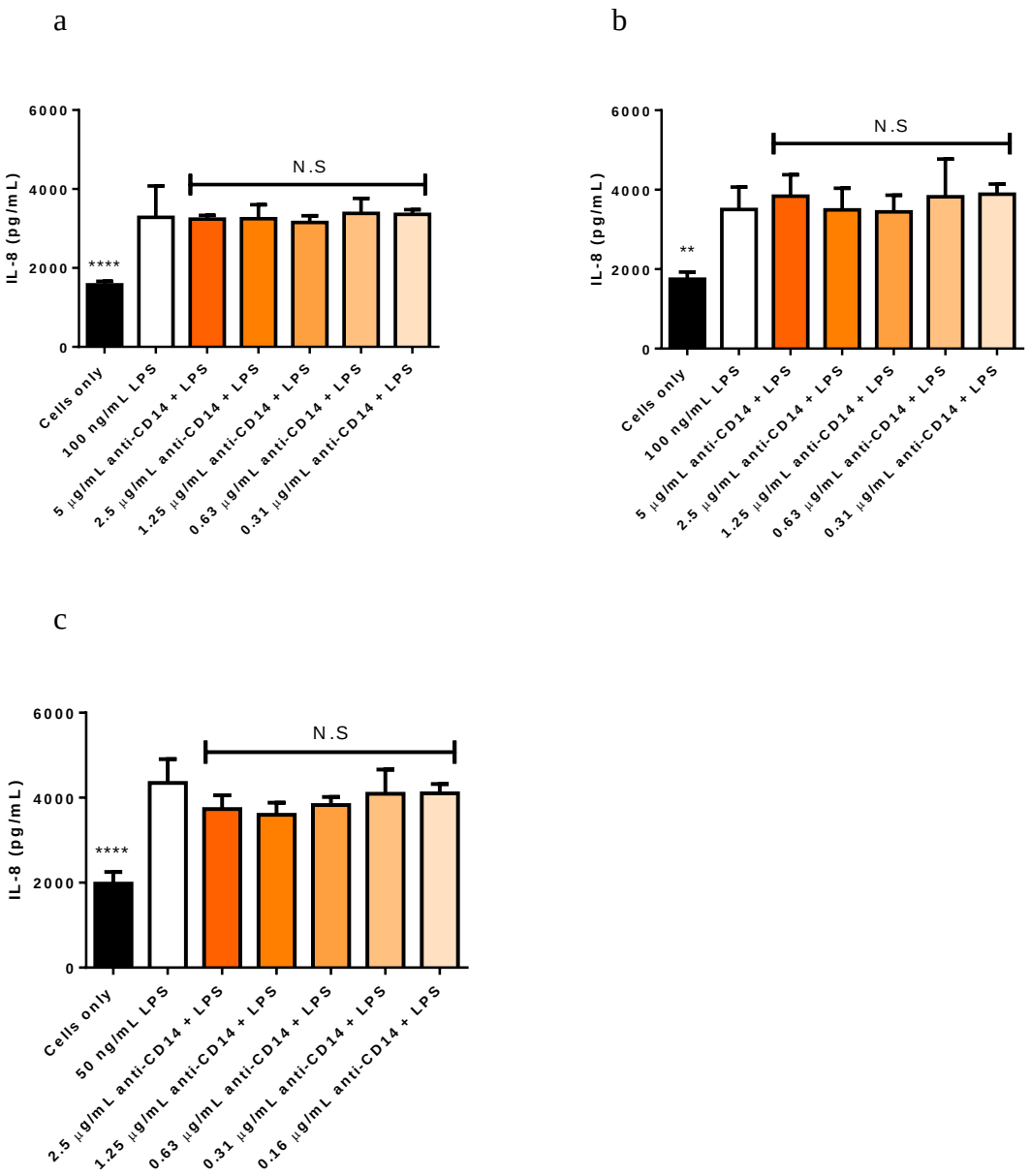


Figure 3.20. IL-8 production in response to anti-TLR4 and LPS. Semi-confluent HT-29 cells were incubated for 1 h (a) or 3 h (b) with 5-0.31 µg/mL anti-TLR4 prior to a 24 h stimulation with 100 ng/mL *E. coli* LPS. Alternatively, HT-29 cells were incubated for 24 h with 2.5-0.16 µg/mL anti-TLR4 during a stimulation with 50 ng/mL *E. coli* LPS (c). Statistical analysis; one way ANOVA with multiple comparisons (Dunnet's multiple comparison test). N.S, not significant; ** $p < 0.01$ and **** $p < 0.0001$ compared to the LPS stimulation alone. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

4.0 Discussion

The etiology of NEC remains unclear and it is thought to arise due to multiple factors[3,30]. Intestinal epithelial cells are increasingly implicated in immunological responses[165] and are particularly important in the pathogenesis of NEC[166]. Probiotics are used clinically in an attempt to prevent NEC and some decreases in disease incidence, mortality and severity have been reported[100,102,167]. The exact mechanism of action of probiotics is not well known[103,168], although some literature suggests an immunological role[107]. For this reason, the immunological response of epithelial cells to certain NEC-associated stimuli and the ability of probiotic bacteria to influence this response was assessed during this study.

4.1 The contribution of enterocytes in the pro-inflammatory response during NEC

4.1.1 LPS and IL-8

IL-8 is produced by enterocytes[169] and is an important chemokine implicated in the inflammatory response, specifically involved in the recruitment and activation of neutrophils[170]. It is hypothesised that an exaggerated inflammatory response that includes excessive IL-8 production, may contribute to NEC progression[21,67,142]. IL-8 production is also correlated to NEC and disease severity, with circulating IL-8 levels higher in the serum of NEC-affected infants compared to control infants[135] and stage 3 infants compared to stage 1 and 2 infants[134]. Particular attention was paid to IL-8 during this study as it is thought to be essential in the induction of the inflammatory response seen in NEC[33,63]. To establish whether intestinal epithelial cells are able to contribute to the immunological response observed during NEC, HT-29 and Caco-2 cells were stimulated with LPS or four different heat-killed *Enterobacteriaceae*, a family of Gram negative bacteria that have previously been associated with NEC[6,88,89]. Specifically, LPS was first used as we wanted to investigate whether HT-29 cells were capable of producing IL-8 in response to a typical inflammatory molecule.

The *C. sakazakii* strains were used as *C. sakazakii* has been previously linked to formula milk contamination and NEC outbreaks[93], and *K. pneumoniae* VIII 8 and *E. cloacae* I 1 were used to see if clinically isolated bacteria (from NEC suspected and NEC confirmed infants respectively) could elicit a response, before investigating a number of clinical isolates.

In response to LPS, a dose and time-dependent increase in IL-8 production was observed (Figure 3.1), a result similar to previous literature[171,172]. Constitutive production of IL-8 was observed by the HT-29 cells and LPS stimulation resulted in chemokine concentrations similar to previous studies (approximately 4500 pg/mL IL-8 in response to 100 ng/mL, compared to 4000 pg/mL in response to 100 ng/mL in the present study)[172]. For later experiments, 100 ng/mL LPS was used as an IL-8 positive control, as it elicited the strongest response and is also close to physiological gut levels[125]. The observation that IECs produce IL-8 in response to LPS also extends to partially and completely differentiated Caco-2 cells (Figure 3.5), other intestinal epithelial cell lines[173] and human infant biopsies[63], ratifying the results in this study. Caco-2 cell differentiation was judged by time dependence and, similar to previous literature, an incubation of 16 days prior to stimulation was considered sufficient to differentiate Caco-2 cells into a polarized monolayer[157]. However, the differentiation was confirmed visually with a microscope and not experimentally tested. Some literature suggests that using tissue culture inserts, applying serum only to the basolateral media and measuring differentiation by assessing monolayer permeability (using trans epithelial electrical resistance (TEER)) and alkaline phosphatase activity is the most effective way to differentiate Caco-2 cells in order to mimic physiological conditions[174], a point that could be addressed in future assays.

As IFN-γ has previously been reported to increase the LPS induced IL-8 production in IECs (from 100 to 400 pg/mL IL-8 in response to 100 ng/mL LPS)[175], IFN-γ was included prior to or during the 24 h LPS stimulation. However, incubation of HT-29 cells with IFN-γ prior to (Appendix 5.2, Figure 5.1) or during the LPS stimulation (Figure 3.1) did not increase the IL-8 production, concordant with other literature[172].

4.1.2 Bacteria and IL-8

It is known that the presence of bacteria is essential for the pathogenesis of NEC, although no single entity is thought to be the cause[19]. The crucial role of bacteria in the disease is indicated by many observations including the presence of bacteraemia, endotoxemia, PI, the effectiveness of antibiotics and probiotics in treatment regimens[66], the inability of NEC to occur *in utero* in the germ-free environment despite fetal ingestion of amniotic molecules[23,67] and the fact that germ-free or colonisation-altered animal models of NEC have an altered or absent NEC pathology or differences in NEC rates[67,168,176]. In light of this, the immunological response of enterocytes after bacterial challenge as measured by IL-8 production was investigated. Prior to undertaking these experiments the growth patterns of four NEC-associated *Enterobacteriaceae* species were determined by measuring the optical densities and calculating the cfu/mL (Figure 3.2 a-d). The growth patterns and bacterial numbers observed reflected similar results obtained in the literature[177-179]. To determine the different growth phases more accurately, samples at more time points would have to be undertaken.

In order to determine the contribution that enterocytes may play in the immune response during NEC in response to bacteria, HT-29 cells were exposed to various concentrations of heat-killed, NEC-associated *Enterobacteriaceae*. A time and dose-dependent increase in IL-8 was observed after a stimulation with heat-killed NEC-associated bacteria (Figure 3.3 and 3.4). The finding that HT-29 cells produce IL-8 in response to bacteria from the *Enterobacteriaceae* family (at approximately similar concentrations) has been reported previously (MOI of 1:10 resulting in approximately 3000 pg/mL IL-8, compared to an MOI of 3.33 resulting in approximately 2000 pg/mL in the present study)[156,180]. However, no literature exists concerning the specific bacteria used in this study and their effect on IL-8 production by IECs. Once again, this observation was duplicated using Caco-2 cells (Figure 3.5). During these assays, heat inactivated bacteria were used in order to avoid the possibility of the bacteria simply killing the HT-29 cells, and heat inactivated bacteria have been used to stimulate intestinal epithelial cells before[181,182]. However, in future assays, determining the effect of live bacteria on the epithelial cells would be desirable and possibly addressed if enterocyte viability is not compromised. The concentrations used in the present study reflect the concentrations of bacteria used to stimulate IECs in previous literature[157,180].

Previous studies have shown that IECs (SW620, HT-29, T84 and Caco-2) are able to produce IL-8 in response to a variety of pro-inflammatory stimuli including TNF-α, IL-1β, LPS and IFN-γ[119,140,171,183]. Together, with the LPS and bacterial stimulation data, this suggests that intestinal epithelial cells are able to respond to bacteria, bacterial LPS and also pro-inflammatory signals and by extension, implicates them in immune surveillance and the pro-inflammatory response seen during NEC.

The HT-29 cell variant, HT-29/MTX, is able to produce mucus and differentiate into mucus producing cells[184], and secretes less IL-8 in response to LPS or IL-1β[185]. This is important as premature infants are thought to have a scanty mucous lining[19,33] and fewer mucus producing goblet cells[47]. Therefore, it would be informative to establish how the HT-29/MTX cell line responded to the *Enterobacteriaceae* used in the current study and to see whether a difference in the IL-8 produced between HT-29 cells and the mucus producing type could be demonstrated. If a difference was observed this would support the idea that the reduced mucus lining seen in the gut of premature infants plays a role in the development of NEC.

IL-8 mRNA is increased in NEC tissue[142] and it has been demonstrated that the fetal human enterocyte cell line (H4 cells) produces more IL-8 when stimulated with *Salmonella enterica*, *E. coli*[186], LPS or IL-1β[63] than adult IECs (T84 or Caco-2). This finding supports the link between prematurity and the risk of developing NEC, because fetal cells are capable of eliciting an inappropriate immune response which may result in tissue damage. Use of the H4 cell line would be advantageous to establish whether these cells would respond with higher IL-8 production than that of adult enterocytes after stimulation with NEC bacteria. This would contribute to the hypothesis that immature enterocytes may initiate an inappropriate immune response. Other studies, where infantile human biopsies were able to demonstrate an increase in IL-8 production by primary immature enterocytes in response to LPS or IL-1β[63], support this theory. The same group also demonstrated that enterocytes from biopsies of immature, undeveloped human ileal tissue had increased IL-8, TLR2, TLR4, MyD88 and NF-κB mRNA levels compared to fully matured tissue[187]. In the current study, further experiments including the utilisation of the H4 fetal enterocyte cell line or the mucus producing HT-29/MTX cells would be informative to demonstrate how the IL-8 production compared.

(N.B. The H4 fetal enterocyte cell line has not been utilised in this study as it is not commercially available and has been developed by a specific lab group[63,188]). Experimental evidence using HT-29 cells, combined with literature reports that immature intestinal epithelial cells during NEC are able to produce IL-8 in response to bacteria, suggests that the premature infant intestine may be predisposed to an excessive immune response.

4.1.3 Clinically isolated bacteria

It was also hypothesised that bacteria isolated from infants with confirmed or suspected NEC would induce the production of more IL-8 by HT-29 cells than those from control group infants. Each isolate caused a significant increase in the IL-8 production when added to HT-29 cells at a concentration of $1x10^8$ cfu/mL compared to the cells only controls (Figure 3.6). However, no significant difference was found between any isolate (irrespective of the infant NEC group) in the ability to cause IL-8 production by HT-29 cells (Appendix 5.2, Figure 5.11). This is in accordance with literature that suggests no single pathogen is the cause of NEC[79,189]. However, this is the first report investigating these bacterial isolates or isolates from control and NEC-affected infants and their differential effect on IL-8 production by IECs. Numerous other bacterial isolates from the different NEC group infant faecal samples are stored, and it would be interesting to utilise these to see if the same trend continued or if a difference in IL-8 production by the HT-29 cells in response to the bacteria from different NEC group infants could be demonstrated. It is predicted that a trend of increased IL-8 production by the HT-29 cells in response to the bacteria isolated from NEC confirmed infants, compared to the NEC control infants, may arise, contributing to the idea of undesirable colonisation.

Taken together, these data along with the bacterial and LPS stimulation experiments show that a number of different clinically isolated, LPS-producing bacteria, and also purified LPS, are able to induce a pro-inflammatory response by HT-29 cells. By extension, this implicates the IL-8 production by enterocytes during the progression of NEC. However, the bacteria isolated from control or NEC-affected infants did not induce differential IL-8 responses, indicating that the pro-inflammatory response initiated after bacterial insult is not necessarily specific to any one organism or *Enterobacteriaceae* strain.

In order to confirm the idea that IL-8 production is essential for NEC, it would be useful to see if mice deficient in the mouse IL-8 homologues KC and MIP-2 had decreased incidence or severity of NEC. Furthermore, mice with defective neutrophils that are unable to be recruited to the site of inflammation during NEC (possibly defective in the IL-8 chemokine receptors CXCL1 or CXCL2) may demonstrate the importance of neutrophil migration to the epithelial barrier[190].

4.2 The immunomodulatory effect of probiotics

The treatment of NEC is mainly supportive with no precise treatment therapy[81]. However, more recently, in order to try and prevent the onset of NEC, probiotic bacteria (including *B. infantis* and *L. acidophilus* in the form of Infloran® in the Dunedin Hospital NICU) have been administered to at risk infants in certain institutions. Probiotics are known to have a number of effects including improving mucosal barrier function, influencing pathogen colonisation, promoting mucus secretion, producing bacteriocins, influencing the gut pH, maintaining tight junctions[81,99,107,168], and are also thought to play immunological roles[81,107]. More specifically, probiotics such as *Lactobacillus* and *Bifidobacterium* spp. in pure cultures, have been shown to reduce IL-8 production by IECs in response to a number of stimuli including TNF-α, IL-1β, LPS and live *Salmonella enteritidis*[155,159].

4.2.1 Heat-killed probiotics

To see if the probiotic bacteria isolated from Infloran® would elicit a pro-inflammatory response, various concentrations of heat-killed probiotic bacteria were incubated with HT-29 cells (Figure 3.8). Unlike the NEC-associated *Enterobacteriaceae* or LPS (Figure 3.1-3.6.), the probiotic bacteria did not induce any significant increase in IL-8 production compared to the cells only control after 24 h, regardless of concentration. This result is similar to those obtained in other studies that found live or heat-killed *Lactobacillus rhamnosus*[144] or live *Bifidobacterium* and *Lactobacillus* spp. did not induce an IL-8 response from IECs[155]. Similar results were found using Caco-2 cells (Figure 3.5). However, unlike other literature, it was found that the baseline IL-8 production was not decreased in response to probiotic challenge[145], although viable and not heat-killed bacteria were used in this case.

Most reductions in inflammatory cytokine production by epithelial cells from the GI tract have been demonstrated *in vitro* with very few studies looking specifically at the IECs *in vivo*[144,191]. However, epithelial cell organ cultures from sections of the ileum or the colon of IL-10 deficient mice showed decreased TNF-α and IFN-γ production when mice were orally administered a mixture of *Bifidobacterium, Lactobacillus* and *Streptococcus* DNA or *B. infantis* conditioned media[192,193]. Despite this, linking the cytokine production and the probiotic influence specifically to the enterocytes themselves is difficult.

In order to test this, administration of oral probiotics to mice could be carried out and ileal and colonic biopsies taken. The epithelial cells could be sorted using flow cytometry, and cultured with a stimulus to determine if there was a difference in the response elicited by enterocytes from probiotic and control treated mice. Alternatively, sections of the mucosa could be taken and stained specifically for enterocytes, and also intracellular cytokines, using fluorescent immunohistochemistry. This would give a definitive answer as to whether it is the enterocytes themselves that the probiotics are acting on. Animal models of NEC do exist, and although they have a number of problems (such as utilising full term and not preterm offspring, inducing NEC prior to substantial bacterial colonisation and the inability to replicate physiologically relevant spontaneous NEC[194]), these same experiments could be conducted in animals under experimental NEC settings. Furthermore, enterocyte stem cells from human intestinal biopsies can be used to create *ex vivo* three dimensional primary intestinal epithelial cell organoids[195]. Using these, in co-cultures with bacteria, the ability of probiotics to reduce the IL-8 production by primary IECs in response to bacterial or LPS stimulation could be demonstrated, confirming the present result.

The observation that probiotic bacteria do not induce IL-8 could be beneficial or detrimental. NEC is associated with increased IL-8 production both locally and systemically[135,142] and it is apparent that increased serum IL-8 ($4x10^4$ pg/mL in stage 3 NEC infants compared to undetected for control, stage 1 and stage 2 NEC infants, eight hours after onset) is associated with increased NEC severity[134], and that excessive inflammation is present during NEC[47,63]. Therefore the fact that probiotics do not elicit a response and do not contribute to the inflammation may be beneficial.

Conversely, IL-8 production may be a good thing locally. It is involved in the attraction and activation of innate immune cells, specifically neutrophils[170]. Neutrophils are important as they are excellent phagocytes and critical for extracellular bacterial clearance[196]. Therefore, it may be hypothesised that more, local IL-8 could be beneficial during an infection. However, most literature agrees that excessive IL-8 production causing inappropriate immune cell recruitment and activation and exaggerated inflammation is detrimental during NEC[19,135]. The ability of the *Enterobacteriaceae* to elicit a strong response by the IECs, and the inability of the probiotics to do so, may also reflect the idea that premature infants have an altered colonisation pattern, which may play a role in the induction of NEC.

Since the probiotic bacteria *B. infantis* and *L. acidophilus* are used clinically to prevent NEC[23,197] and probiotics have been shown to influence cytokine production previously[107,144,159], it was decided to see if they could play an immunomodulatory role in the present study. HT-29 cells were either pre-treated with probiotic bacteria and then stimulated with NEC-associated bacteria or LPS or co-incubated with probiotics and NEC-associated bacteria or LPS, to see if a reduced IL-8 production ensued. The IL-8 production by HT-29 cells was unable to be reduced under any treatment condition by heat-killed *B. infantis* or *L. acidophilus* (Figure 3.9, Appendix 5.2, Figure 5.2-5.5).

A number of reasons are hypothesised for why this may be, despite other literature achieving a reduction in IL-8. These include the probiotic concentrations, the duration of the probiotic prime, the duration of the stimulus and possibly the type of stimulus used (as it is though that these factors may impact the ability of the probiotics to reduce pro-inflammatory signalling). However, probiotic pre-treatments of IECs for just one to two hours and up to 12 hours have worked previously to reduce inflammatory responses[144,145,156,157]. Some studies have shown that the probiotic bacteria need to be intact and alive to exert their benefit[158,198], although inhibition of inflammatory responses by probiotic cell debris, fractions, cell constituents, heat-killed bacteria or conditioned media is also documented[144,151,155,157].

The concentrations of probiotic bacteria used in other papers ($1x10^6$, $1x10^7$ and $1x10^8$ cfu/mL) that resulted in significant decreases in inflammatory responses are similar to the concentrations used in our study ($1x10^6$, $1x10^7$ and $1x10^8$ cfu/mL)[144,145,155]. Therefore, we do not think that the inability of the probiotic bacteria to reduce the IL-8 production in this case is because of the concentrations used. Taken together this data indicates that, although heat-killed probiotics do not induce a pro-inflammatory response, they were unable to reduce it in the presence of *Enterobacteriaceae* or LPS under the experimental conditions here. This leads to two questions; is there any way we can reduce the pro-inflammatory response of HT-29 cells and what is the effect of live probiotic bacteria?

4.2.2 LTA

To address the first question, it was decided to see if a component of the Gram positive cell wall lipoteichoic acid could reduce the IL-8 production by HT-29 cells as seen in other literature[151]. It was found that a co-incubation of a high concentration of LTA (100 µg/mL) was able to reduce the amount of IL-8 produced in response to LPS (Figure 3.10). This finding is in accordance with other literature that showed the TLR-2 agonist, LTA, is able to reduce the pro-inflammatory response elicited by LPS at approximately similar concentrations (10-100 µg/mL of LTA)[151]. Although it is not well understood why this reduction occurs, it is thought it may be due to the ability of LTA to block the binding of LPS to the CD14 molecule, as they share the same co-receptor[151]. Another study found that LTA from *Lactobacillus plantarum* reduced *Shigella flexneri* peptidoglycan induced TNF-α and IL-1β production by THP-1 monocytes, and that this tolerance was mediated by the down regulation of NOD2 expression[199]. It has been shown that LTA may also have pro-inflammatory effects on immune cells such as monocytes[152,153]. However, LTA does not stimulate IL-8 production or NF-κB activation by IECs due to their minimal expression of TLR2[200]. This suggests that LTA is unable to induce an inflammatory response by enterocytes and that it may be able to reduce the enterocyte immunological response caused by LPS. Although further investigation is warranted, immunomodulation by LTA may be one reason for the probiotic bacterial efficacy in reducing the incidence and severity of NEC.

This highlights the point that despite *B. infantis* and *L. acidophilus* both having this component, as heat-killed entities, they were unable to reduce the IL-8 response. Reasons for this could be that the LTA is not produced in high enough concentrations during these assays by the probiotics or that the LTA is destroyed by the heat inactivation process. However, a previous study demonstrated that LTA from *Streptococcus pyogenes* is heat stable and remained functional after a substantial heat treatment (95°C for 30 min)[201]. It is important to note here that although LTA was able to reduce the IL-8 in response to LPS, we do not know the mechanism. Furthermore, we do not know whether this is the only component of probiotics or Gram positive bacteria that may do so. Another interesting point is that the baseline IL-8 production remained unchanged (Appendix 5.2, Figure 5.13), indicating that LTA blocks the signalling of the pro-inflammatory response and does not simply non-specifically reduce IL-8 production. The concentrations of LTA used in the current study reflected the amounts used in previous literature[151]. Interestingly, it has been shown previously that LTA may be able to interact with the LPS co-receptor, CD14[151-153]. The interaction between LTA and CD14 may account for the reduction in LPS induced IL-8 production seen here. LTA is predominately expressed by Gram positive bacteria, which are commonly used as probiotics, e.g. *B. infantis* and *L. acidophilus* in Infloran®. The fact that LTA from Gram positive bacteria may reduce IL-8 production due to the inhibition of LPS signalling via CD14 may be a mechanism by which NEC reductions are seen when probiotic supplementation is used. An exciting experiment would be to see if purified LTA could decrease the incidence or severity of NEC in a rat or mouse model of NEC, although no studies exist on the matter. Also, it would be interesting to see if the ability of LTA to bind CD14 or reduce NOD2 expression could be demonstrated here, in order to establish a mechanism.

It should be mentioned that it is well established that LTA molecules also have pro-inflammatory effects on innate immune cells[149,150]. Therefore, its ability to reduce IL-8 in response to LPS by HT-29 cells may not result in a protective effect during NEC. However, the structure of LTAs from different species show great diversity and therefore LTAs from certain species may be pro-inflammatory while others may be anti-inflammatory[151]. Also is thought that the pro-inflammatory effects seen in certain studies were due to LPS contamination of LTA[151].

In the current study, pure LTA and not heat-killed probiotic Gram positive bacteria were able to reduce the amount of IL-8 in response to LPS. Possibly LTA is destroyed or removed during the heat inactivation process. A bacterial inactivation process that is gentler than heat inactivation, such as gamma or ultra-violet irradiation could be utilised to ensure a minimal amount of bacterial proteins are disrupted.

4.2.3 Live probiotics

It was decided to see if live *B. infantis* or *L. acidophilus* could reduce the IL-8 production by HT-29 cells in response to LPS. Heat-killed probiotic bacteria were used prior to this as it was hypothesised that the lactic acid produced by the bacteria would kill the HT-29 cells.

In contrast to heat-killed bacteria (Figure 3.11 and Appendix 5.2, Figure 5.3) it was found that live *B. infantis* could reduce the IL-8 production by HT-29 cells during an LPS challenge (Figure 3.11). This finding is in line with other literature that suggests probiotic bacteria (live, dead or components) can have an immunological role and reduce pro-inflammatory signalling by enterocytes[144,155,158]. It was found that live *L. acidophilus* was unable to reduce the IL-8 production, an observation that is difficult to explain as previous studies have shown a reduction in response to LPS, *S. enterica*, IL-1β or TNF-α in IECs[144,155,158]. The fact that the strains of *Lactobacillus* utilised were different species or obtained from different sources may explain this. It is known that different species and also strains of probiotics have different immunomodulatory effects and may differ in their mechanism of action[99,156], and possibly the strain from Infloran® that we utilised is unable to reduce IL-8 production by IECs. It should be noted here that the probiotics utilised during this study are anaerobic bacteria and would not remain viable for an extended period of time outside anaerobic conditions. Also, during prophylactic probiotic treatment, the probiotic bacteria are administered at the same time, therefore the ability of the bacteria to synergistically reduce the IL-8 response should be investigated. Also, the live probiotic bacteria were not adjusted to any specific growth phase prior to the use in these assays and it is possible that the probiotics have different effects during different growth phases, an idea that could be addressed in the future. Live probiotic treatment also resulted in reduced HT-29 cell viability, this may be addressed by using conditioned media in order to investigate soluble proteins.

Also, despite that the NEC bacteria utilised were heat killed, differing growth phases of heat killed bacteria have previously effected cytokine production[202]. Therefore, it would be informative to investigate the ability of different growth phases of NEC bacteria and also probiotic bacteria to influence cytokine production.

Although some molecular mechanisms for the immunological effects of probiotics on enterocytes have been demonstrated (NF-κB regulation, mitogen-activated protein kinase (MAPK) regulation and IκB stabilisation), many mechanisms and the specific probiotic molecules involved remain unknown[203]. Some studies have suggested that the reduction in IL-8 production caused by probiotics in IECs due to a pro-inflammatory stimulus, may be due to increased IκBα stability, the reduction of NF-κB signalling and decreases in IL-8 mRNA[144,158]. Another study demonstrated that components of probiotic bacteria such as LTA may block LPS signalling[151]. Also, it has been shown that the probiotic bacteria *L. casei* could reduce *Shigella flexneri* induced IκBα degradation and also TNF-α induced NF-κB activation in human kidney cells[108]. Furthermore, structural and soluble components of three different *Lactobacillus* species were able to reduce the TNF-α induced IL-8 production by Caco-2 cells, although the mechanisms were not identified[157]. Additionally, it has been shown that the ability of the probiotic bacteria *B. infantis*, *L. casei* and *L. lactis* to reduce IL-8 production by Caco-2 cells in response to *Salmonella enterica* may have been due to their ability to induce the expression of heat shock protein 70 (Hsp-70), a protein with protective effects in IECs[159]. In light of this, it would interesting to see if the Hsp70, IκBα or NF-κB expression could be influenced by *B. infantis*, giving a possible mechanisms of action for the IL-8 reduction.

The data from the current study demonstrates that probiotics can be immunomodulatory and may be able to play an immunological role in the intestinal environment of a premature neonate in order to prevent NEC. Probiotics have many roles in the gut mucosa other than immunological[99] and the capacity of probiotics to reduce IEC IL-8 production is probably just one aspect of what they do. It should be mentioned here that caution should be taken when administering probiotics[204]. It is possible that probiotic treatment may lead to sepsis in isolated cases of immunocompromised hosts and that unexpected and long term health effects may occur, although they are widely unknown.

Furthermore, NEC occurs in the most fragile infants, therefore caution should be taken and extensive research must be undertaken to fully understand the impacts of probiotics[204].

Administration of infants with human milk results in fewer cases of NEC compared to feeding infants with formula milk[205,206]. It has been shown that factors such as TGF-β and erythropoietin in human milk can reduce the inflammatory response of epithelial cells (including IL-8 production) and specifically fetal IECs after a pro-inflammatory challenge[143]. It is also known that breast fed infants are more likely to be colonised with bifidobacteria (as opposed to *Enterobacteriaceae* in formula fed infants)[207,208] and have a higher diversity of *Bifidobacterium* spp.[209]. It has also been found that breast milk actually contains *Bifidobacterium* spp. along with special 'bifidogenic oligosaccharides' that enhance the ability of bifidobacteria to grow[210,211], further contributing to the favourable colonisation of the infantile GI tract. Human milk further contributes to the infantile GI tract defences as it contains secretory IgA, which is hypothesised to help prevent microbial intestinal infections[55]. TLR4 is important in the induction of NEC and it is thought that human milk may be able to reduce the expression of TLR4[212], although the mechanism is not explained. Together, the ability of milk to reduce inflammation, increase probiotic bacterial colonisation and also reduce the expression of TLR4 may explain the protective effect of human milk against NEC.

4.3 The role of CD14 and TLR4 during NEC

It is known that TLR4 is essential for the pathogenesis of NEC[22,112]. However, the role of the TLR4 co-receptor, CD14, is not well characterised. CD14 can be membrane-bound or secreted and its role is to present LPS to MD-2 and TLR4[111,213]. It has been shown that enterocytes, including HT-29 cells, express CD14[119,125,171]. However, it is not very well understood whether CD14 plays an important role in LPS signalling in enterocytes. During NEC there is an increase in CD14 expression and it is thought that this may play a role in the development of NEC[37,120]. In a rat model of NEC, administration of anti-CD14 antibodies resulted in a decrease in inflammation and mucosal disruption as well as a reduction in serum TNF-α and IL-6 levels[37], further implicating the LPS co-receptor.

In order to detect CD14 on HT-29 cells, flow cytometry was utilised (Figure 3.12) as enterocyte CD14 expression has been demonstrated by flow cytometry previously[119]. In the first experiments conducted, the isotype control antibody indicated non-specific binding of antibodies was occurring. Therefore, this issue was addressed before gathering data (Figure 3.13). After changing the antibodies to those conjugated with a different fluorochrome (brilliant violet instead of PE), it was found that the non-specific background signal was no longer an issue (Figure 3.13 c). We hypothesise that the PE isotypes previously utilised may have been too bright or conjugated to more fluorochromes than the primary antibody, thus making the isotype signal stronger, indicating that non-specific binding was occurring (false positives).

A CD14 positive control was also needed to ensure CD14 could be detected reliably. THP-1 cells have been shown to express CD14[161] and are often used as a positive control for CD14 during flow cytometry experiments[162,163]. However, it was found that neither differentiated nor undifferentiated THP-1 cells expressed CD14 at detectable levels (Figure 3.14 a). This may be due to THP-1 CD14 existing in a conformation not detected by the antibody utilised. PBMCs are known to express CD14[164] and have been used as a CD14 positive control previously[119]. PBMCs gave high levels of CD14 expression (Figure 3.14 b) and were used as the CD14 positive control in future experiments. THP-1 cells were not further investigated as a positive control because PBMCs expressed high levels of CD14 and were a suitable control.

4.3.1 The influence of LPS on CD14 expression

CD14 can be up regulated in response to LPS in certain cells such as monocytes, macrophages[214,215] and enterocytes[120,125]. Furthermore, it has been found that CD14, TLR4 and MD-2 are up regulated in human NEC tissue[37]. The CD14 expression by HT-29 cells was tested in response to different concentrations of LPS. Despite the PBMC positive control showing CD14 was detectable, CD14 was undetectable on HT-29 cells, regardless of LPS stimulation (Figure 3.15). This finding is in line with reports in the literature that demonstrated CD14 was undetectable on certain enterocytes using flow cytometry[175,216]. However, CD14 has been detected in HT-29 cell using flow cytometry previously[119,162]. Although the methods used in the current study were similar to those used previously, CD14 was undetectable.

Another IEC line that is reported to express CD14 is the Caco-2 cell line[125]. However, CD14 was also undetectable on these cells (Figure 3.16 and Appendix 5.2, Figure 5.12). A possible explanation for the inability of flow cytometry to detect CD14 on the IECs is that the CD14 molecule on the HT-29 or Caco-2 cells may be in a slightly different conformation (and different forms of CD14 with structural differences do exist[217]) than that of the PBMCs, and the antibody clone being utilised could not bind efficiently to the CD14 molecule. Therefore, the use of a different antibody clone (as was done in the Western blot) may be able to detect the CD14. Another possibility is that the HT-29 or Caco-2 cells were expressing CD14 at much lower levels than the PBMCs and was therefore undetectable. Soluble forms of the protein do exist[119,217,218] and possibly the CD14 molecule is released by the enterocytes and therefore not detected. Intracellular staining of HT-29 cells was carried out to try and detect soluble CD14 before its release, however, no reliable data was obtained. ELISAs that detect soluble CD14 also exist, and in the future, may be implemented to detect secreted CD14. As CD14 was undetectable by flow cytometry Western blotting was used.

4.3.2 CD14 and TLR4 expression

Western blotting detected CD14 protein in the PBMC positive control and also the HT-29 cells (Figure 3.17). One CD14 band was found in the PBMC positive control, while HT-29 cells showed the presence of two CD14 bands (54 and 48 kDa). Multiple forms of CD14 have been seen before[217] and those seen here may correspond to a membrane bound and a soluble form of CD14[217], or two different soluble forms[219]. It was found that LPS at the concentrations used did not cause a significant increase in CD14 expression, a finding that is supported by a previous study that used similar concentrations of LPS[119]. It would be interesting to see if the increase in CD14 expression by Caco-2 cells or intestinal epithelial cells *in vivo* seen in previous literature[125] could be replicated. The reason CD14 was detected by Western blot and not flow cytometry is not known. We hypothesise that it may be due to receptor secretion, different protein folding, assay sensitivity or the use of a different antibody clone.

TLR4 is the putative LPS receptor[111] and it has been shown to be expressed by enterocytes including HT-29 cells[121,216]. Activation of TLR4 by LPS results in NF-κB activation, gene transcription and cytokine production[220,221].

TLR4 is implicated in the development of NEC[37,112] and an animal model of NEC has shown that TLR4 mRNA is increased in the crypts and villi of ileal samples of formula fed, hypoxia stressed neonatal rats[67]. Also, along with a number of other toll like receptors, TLR4 mRNA and protein levels are up regulated in the ileum of mice with NEC, compared to non-NEC controls[126] and mutations to the TLR4 gene protected mice from developing NEC[22]. Previously, LPS has been shown to increase the IEC expression of TLR4 and it has been demonstrated that administration of intraperitoneal LPS to mice causes an increase in TLR4 expression by the intestinal epithelium[125].

TLR4 protein expression was detected by Western blot, and a band corresponding to TLR4 was evident at approximately the correct molecular weight (95 kDa) in both PBMCs and HT-29 cells (Figure 3.18). However, the expression levels of TLR4 on the HT-29 cells did not change with LPS stimulation, regardless of the concentrations used (Figure 3.18). The concentrations of LPS used here (1, 10, 100 and 1000 ng/mL) were similar to those used in a previous study (300 ng/mL) that showed LPS can influence the TLR4 expression in IECs[125]. However, Caco-2 and primary mouse IECs were investigated during this study, and not HT-29 cells. Therefore, it would be interesting to see if the result seen previously could be replicated using Caco-2 cells or mice.

Taken together, this data shows that HT-29 cells express the LPS receptor, TLR4 and also the LPS co-receptor CD14, a finding that is confirmed by previous literature[171,175]. However the LPS stimulation did not affect their relative expression. Earlier, we showed that NEC-associated bacteria caused an increase in IL-8 production by HT-29 cells, sometimes greater than that of the LPS positive control (Figure 3.3 a). Furthermore, bacteria have previously been shown to alter toll like receptor expression in certain human cells[222,223]. Therefore, it would be interesting to investigate whether any of the bacteria utilised during this study could alter the expression pattern of CD14 or TLR4 in HT-29 cells. Also, TLR4 activation has been shown to increase apoptosis and decrease the migration of enterocytes[166] and it would be interesting to see if this could be demonstrated in the HT-29 cells, as impaired restitution, increased apoptosis and poor enterocyte migration is implicated in NEC.

The observation that IECs do not increase CD14 or TLR4 expression after an LPS stimulation is probably beneficial, as it may lead to increased LPS sensitivity and chronic immune activation. However, immature, hyper-responsive IECs have shown to have increased CD14, TLR4, MyD88 and NF-κB mRNA, supporting the hypothesis that immature enterocytes are predisposed to a pro-inflammatory response and that this is important in the induction of NEC[187].

4.3.3 HT-29 cells require CD14 for LPS responsiveness

The finding that HT-29 cells express CD14 does not necessarily mean that they require CD14 for LPS signalling. Furthermore CD14-independent signalling in enterocytes has been shown before[216].

To demonstrate IEC dependence on CD14 for endotoxin recognition, HT-29 cells were incubated with an anti-CD14 antibody during an LPS stimulation. It was found that a co-incubation with anti-CD14 resulted in decreased IL-8 production in response to LPS (Figure 3.19 c). Due to limiting amounts of blocking antibody, only half of the original concentration of anti-CD14 antibody and LPS were used during the co-incubation experiment than in the pre-incubation experiment. This result indicates that CD14 is, at least in part, required for HT-29 stimulation by LPS. It has previously been shown that HT-29 and SW620 cells require soluble CD14 in order to respond to LPS or whole *E. coli*[224] and that MAP kinase phosphorylation and cytokine production in the ileal mucosa in response to LPS requires the presence of CD14 *in vitro* and *in vivo*[120]. However CD14-independent LPS signalling has been shown in Caco-2 and HT-29 cells before[171]. This result has important implications in NEC, as it has been observed that CD14 expression is increased in NEC tissues[37] and IECs during NEC[120].

If enterocytes require CD14 to respond to LPS (as shown in the current study) and CD14 can increase the ability of certain cells to respond to LPS, the presence of increased amounts of CD14 in NEC tissues and possibly IECs may result in an increased sensitivity of the gut mucosa and IECs to LPS and partially explain the increased inflammatory response seen in premature infants leading to NEC. However, it is also possible that the increased CD14 in NEC-affected tissue is a result of the inflammation, and not a cause of it.

In order to support the idea that CD14 expression on IECs is required for the IL-8 production in response to LPS, it would be beneficial to utilise different blocking antibody clones, confirm the results in different IECs and utilise methods such as RNA interference to knock down CD14 expression. Also, to investigate the role of CD14 on IECs during NEC, an animal model of NEC with detrimental mutations to IEC CD14 genes could be utilised. It would also be interesting to see if blocking the CD14 receptor would have an effect on the IL-8 production by HT-29 cells during the *Enterobacteriaceae* stimulation, an idea that would be investigated in future assays.

Previously, it has been shown that TLR4 is required for IEC LPS responsiveness[171,225] and it is apparent that CD14 does not have a transmembrane or signalling domain, and therefore requires another factor to signal intracellularly[111]. However, it was found that in contrast to the CD14 block, antibodies specific to TLR4 were insufficient to reduce the LPS-induced IL-8 production independent of concentration, prime or co-incubation (Figure 3.20). There are a number of factors that could explain this result. Firstly, the antibody used here was the primary antibody used for the TLR4 Western blot, and the use of this antibody for functional *in vitro* studies was not stated by the manufacturer. Antibodies in a biological setting can have many effects such as activation, inhibition or no effect at all, and the antibody utilised may have not occupied the correct site to stop LPS-TLR4 interaction. Secondly, the concentrations used were based on the concentrations used during the CD14 block and the biological activity of these molecules may be different. Therefore a titration and time course would need to be conducted using antibodies generated for *in vitro* assays (as was done for the CD14 block (data not shown)) in order to ensure concentration and timing was correct. Once again it would be beneficial to confirm this result by using different antibody clones, an antibody specific for *in vitro* functional studies, different IECs and methods such as RNA interference in order to show TLR4-dependent signalling. It would also be interesting to see if blocking the TLR4 receptor reduced the IL-8 production in response to the heat killed *Enterobacteriaceae*, a point that could be addressed in the future.

Mutations to TLR4 have previously shown to protect mice against NEC[22], while CD14 neutralizing antibodies administered into the jejunum of rats caused a reduction in NEC pathology, jejunum macrophage infiltration and serum TNF-α and IL-6 levels in a rat model of NEC[37]. Moreover, milk[212] and probiotic conditioned media[107] have previously demonstrated an ability to decrease TLR4 expression in enterocytes and a villin-cre knockout system demonstrated that the TLR4 on enterocytes was important in the induction of NEC[112]. Interference with these receptors may provide a treatment option for NEC and it would be interesting to see if antibodies to both TLR4 and CD14 could prevent NEC in animal models. It was demonstrated here that HT-29 cells express TLR4 and CD14 and that CD14 was required for HT-29 IL-8 production in response to LPS.

4.4 Conclusion

It is thought that the epithelial cells of the infant intestine produce an inappropriate pro-inflammatory response to bacteria during NEC, characterised by excessive IL-8 production. Also, unfavourable colonisation of the premature GI tract and an over-representation of *Enterobacteriaceae* have been implicated in the disease. Probiotics are implemented in an effort to reduce the severity and incidence of NEC and some reductions have been seen. It is thought that probiotics may be able to influence the inflammatory response and reduce the attachment of *Enterobacteriaceae*. The aim of this study was to better understand how enterocytes contribute to the immune response during NEC after bacterial colonisation. We also wanted to investigate the ability of probiotic bacteria to reduce the inflammatory response seen by enterocytes after stimulation and identify the mechanism.

Using HT-29 cells as a model intestinal epithelial cell, it was found that enterocytes produce IL-8 in response to LPS and NEC-associated *Enterobacteriaceae* and that the LPS induction of IL-8 was CD14-dependent. The requirement for TLR4 was not able to be demonstrated. It was established that the probiotics from Infloran® did not induce a pro-inflammatory response and that the IL-8 production induced by LPS was decreased with live *B. infantis*. It was also found that LTA, a component of Gram positive bacterial cell walls was also able to reduce the IL-8 production in response to LPS, possibly through the interaction with CD14.

Ideally, the fetal intestinal epithelial cell line H4 would be implemented in future assays, as it more closely resembles immature IECs. Also, it would be interesting to compare the results gathered here with the mucus producing HT-29/MTX cells, as premature neonates have a scanty mucus lining. The ability of additional clinically isolated bacteria would also be investigated in order to make more substantial conclusions on their capacity to induce IL-8 production. More intensive investigation into the mechanism of action of the probiotics would also be undertaken, in order to confirm the immunomodulatory role they may play in preventing NEC.

From the results gathered in this study, a novel mechanism of action by probiotic bacteria in the prevention of NEC is hypothesised. It is proposed that probiotic bacteria are able reduce the LPS induced IL-8 response by IECs, possibly due to the interruption of the LPS-CD14 interaction by LTA. Therefore, LPS is unable to elicit an IL-8 response by the IECs. This results in the inability of neutrophils to be recruited to the site of inflammation and, following this, reduced inflammatory molecule production occurs. Furthermore, reduced tissue destruction, barrier dysfunction and bacterial translocation would occur, due to a reduction in pro-inflammatory mediators. The proposed mechanism of action is highlighted in Figure 4.1. Despite this, additional intensive investigation is needed to confirm the mechanism. This study is the first to investigate the ability of clinically-isolated bacteria from different NEC group infants to elicit a pro-inflammatory response by IECs. Furthermore, this is the first report investigating the immunological role that the probiotic bacteria from Infloran® play in preventing NEC.

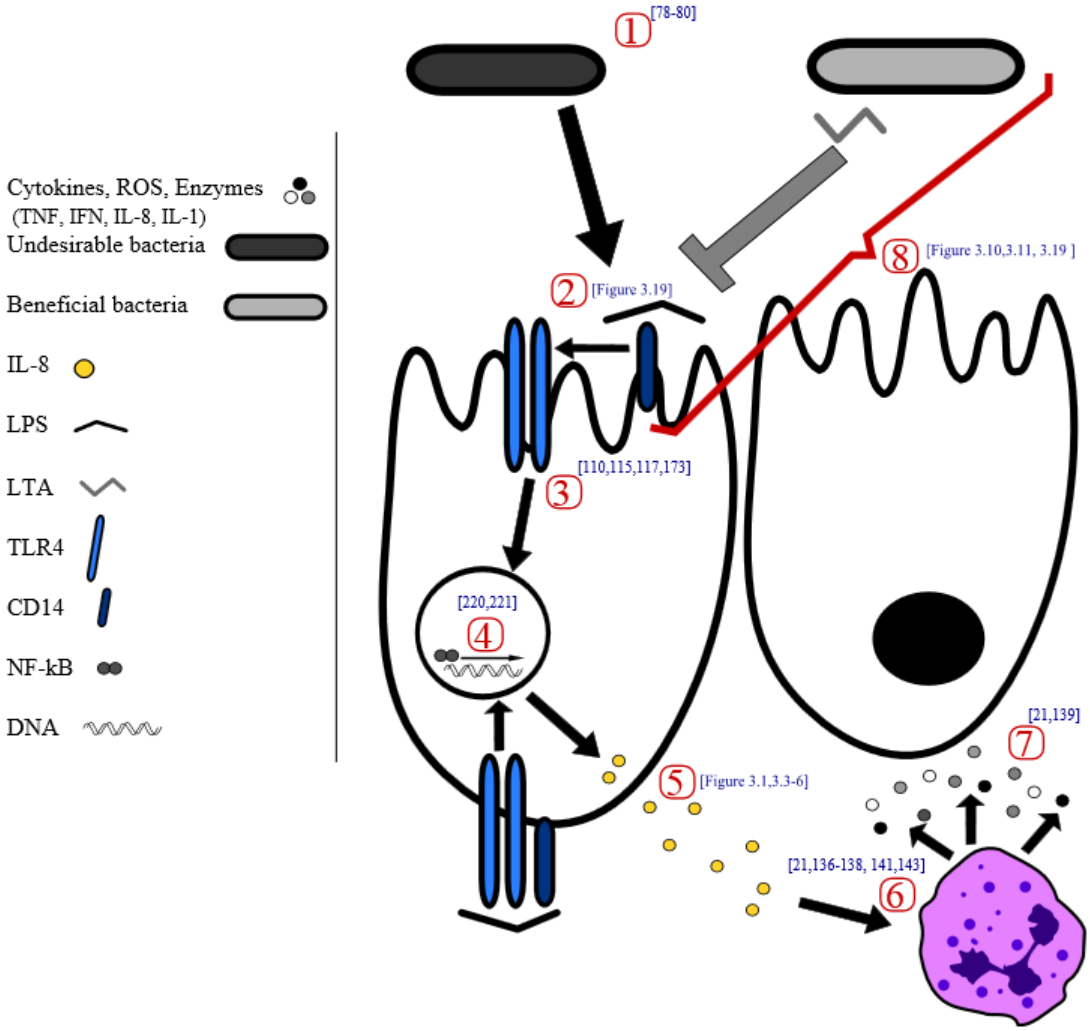


Figure 4.1. The immunological role of enterocytes and probiotics in NEC. Premature neonates have an undesirable colonisation pattern (1). It was shown that enterocytes elicit a response to bacterial LPS in a CD14-dependent manner (2). TLR4 activation in response to LPS and bacteria leads to signalling cascades that may be MyD88-dependent or independent (3), NF-κB translocation and inflammatory gene production in enterocytes (4). In response to NEC-associated stimuli (*Enterobacteriaceae* and LPS), HT-29 and Caco-2 IECs produced IL-8 (5), a chemokine responsible for neutrophil migration and activation (6). Production of pro-inflammatory cytokines, chemokines, ROS and enzymes by neutrophils have deleterious effects on the mucosal barrier (7) and compound the existing defects that result in a disrupted and leaky barrier. Live *B. infantis*, possibly through the action of LTA, present in Gram positive bacteria, interrupt this process, possibly due to the interaction of LTA with CD14 (8). Probiotics play many roles in the prevention of NEC. However, this is an important step as it initiates the ensuing immune response.

5.0 Appendix

5.1 Reagents

PBS: 10 x phosphate buffered saline (PBS) concentrate: 8% NaCl (Scharlau, Spain), 0.2% KCl (Sigma®, USA), 11% Na_2HPO_4 (Sigma®, USA) and 0.2% KH_2PO_4 (AnalaR® BDH, England) in Distilled H_2O. Dilute 1:10 in distilled H_2O and adjust pH to 7.2 ± 0.2 before use (1 x PBS).

5.1.1 Flow cytometry

FACS buffer: Filter sterilised 1 x PBS, 1% FCS (BioInternational, New Zealand) and 0.01% NaN_3 (J.T.Baker, USA).

5.1.2 ELISA

Wash buffer: 1 x PBS, 0.05% Tween-20 (Sigma-Aldrich®, USA).

Coating buffer: Deionized water, 0.15% Na_2CO_3 (AnalaR® BDH, England) and 0.3% $NaHCO_3$, (Emsure® Merck, USA).

Assay diluent: 1 x PBS, 1% Bovine serum albumin (BSA), (GibcoBRL, Life Technologies, USA).

5.2.3 Western blot

Sample buffer: 120 mM Tris buffered saline (pH 6.8, HCl), 5% Sodium dodecyl sulphate (SDS) (Sigma®, USA), 20% Glycerol (Merck, Germany), 0.01% bromophenol blue (BDH® Supplies, England), 10% 2-ME (Gibco®, Life Technologies, USA).

2 x 10 % SDS-PAGE gels:
I Resolving: 3.75 mL acrylamide (40%), 3.75 mL resolving buffer, 7.5 mL milliQ H_20 (NANOPure Infinity ™, Barnstead, USA), 75 µL ammonium persulphate, 15 µL TEMED (Sigma, USA).

II Stacking: 0.5 mL acrylamide (40%), 2.5 mL stacking buffer, 2 mL milliQ H_2O (NANOPure Infinity ™, Barnstead), 35 µL ammonium persulphate, 7 µL TEMED (Sigma).

10 x electrophoresis running buffer: 14.4 % Glycine, 3% Tris, 1% SDS in distilled H_2O, diluted 1: 10 before use in distilled H_2O.

Resolving buffer: 1.5 M Tris, pH 8.8, 0.4% SDS

Stacking buffer: 0.5 M Tris, pH 6.8, 0.4% SDS

5.2.4 Western blot semi-dry transfer

Anode buffer I: 0.3 M Tris, 10% methanol, pH 10.4

Anode buffer II: 25 mM Tris, 10% methanol

Cathode buffer: 25 mM Tris, 40 mM glycine, 10% methanol pH 9.4

5.2 Additional figures

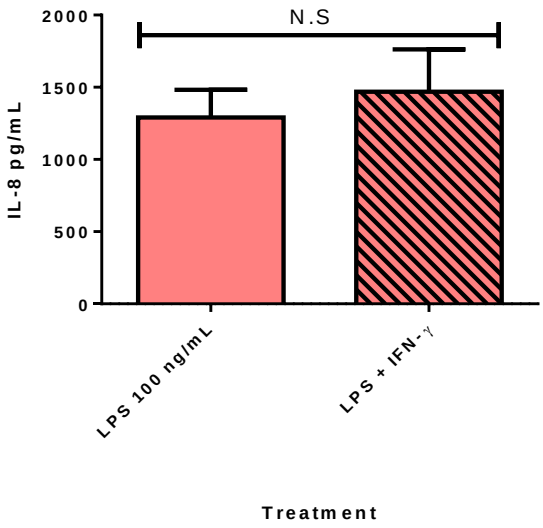
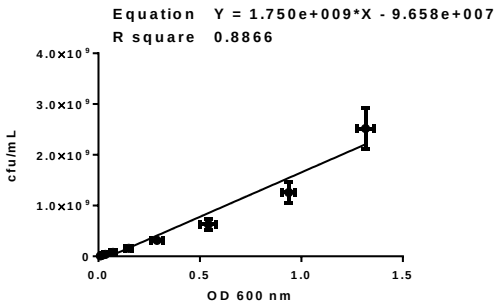


Figure 5.1. IL-8 production in response to an IFN-γ prime. HT-29 cells were incubated with 10 ng/mL IFN-γ for 12 h prior to a wash and the addition of 100 ng/mL LPS for 24 hours. Statistical analysis; un-paired two-tailed T test. N.S, not significant. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments.

a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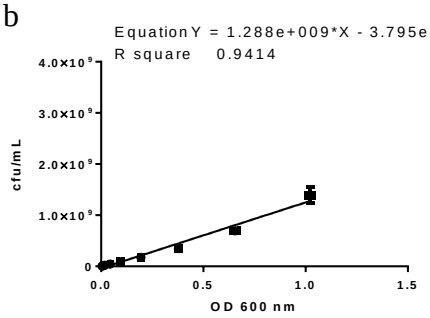
b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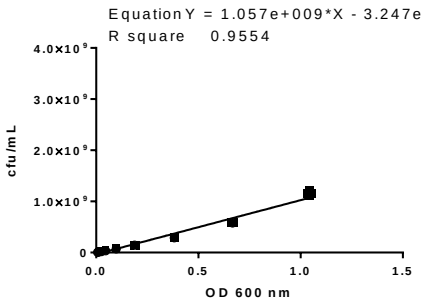
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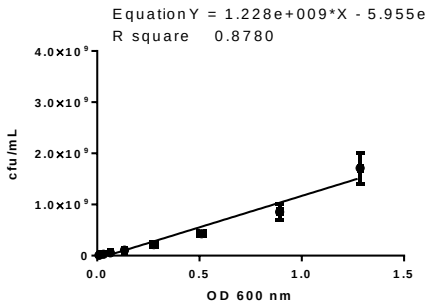
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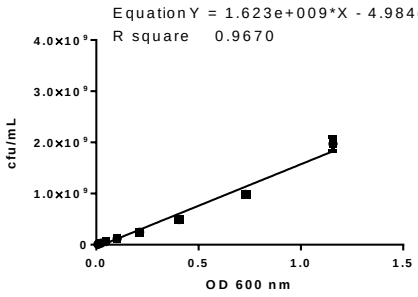
e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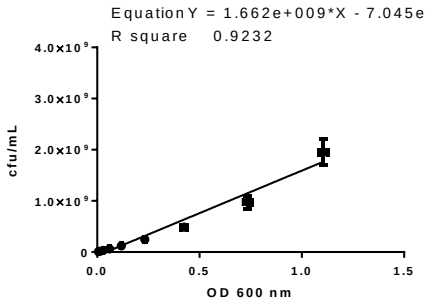
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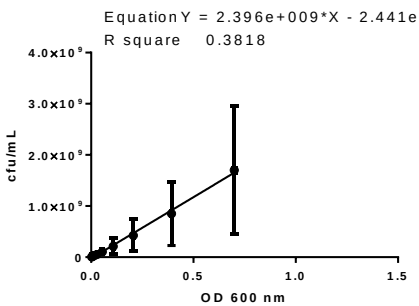
g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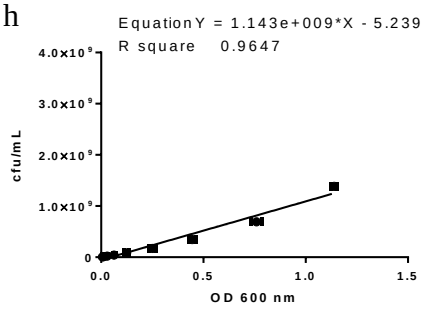
h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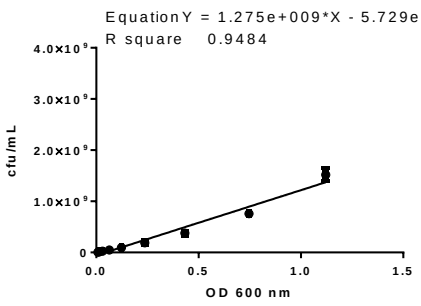
cfu/mL
OD 600 nm

i


Equation Y = 1.275e+009*X - 5.729e+007
R square 0.9484
cfu/mL
OD 600 nm

j

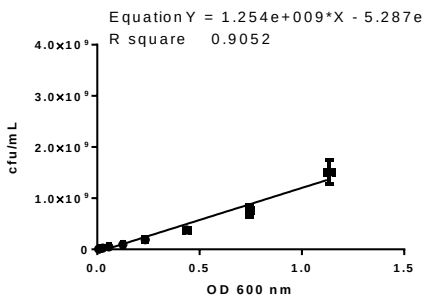

Equation Y = 1.254e+009*X - 5.287e+007
R square 0.9052
cfu/mL
OD 600 nm

Figure 5.2. Bacterial estimation for 10 *Enterobacteriaceae* strains. Bacteria; *K. pneumoniae* (a), *K. oxytoca* (b), *K. oxytoca* (c), *E. coli* (d), *S. marcescens* (e), *K. oxytoca* (f), *R. ornithinolytica* (g), *K. oxytoca* (h), *K. oxytoca* (i) and *K. oxytoca* (j) were incubated for 24 h, 37°C in TSB. Colony forming units were determined using tenfold serial dilutions and optical densities of two fold serial dilutions were read at 600 nm. Data points and error bars represent the pooled mean and SEM respectively of both optical density and cfu/mL. The equation and R^2 value is depicted for each isolate.

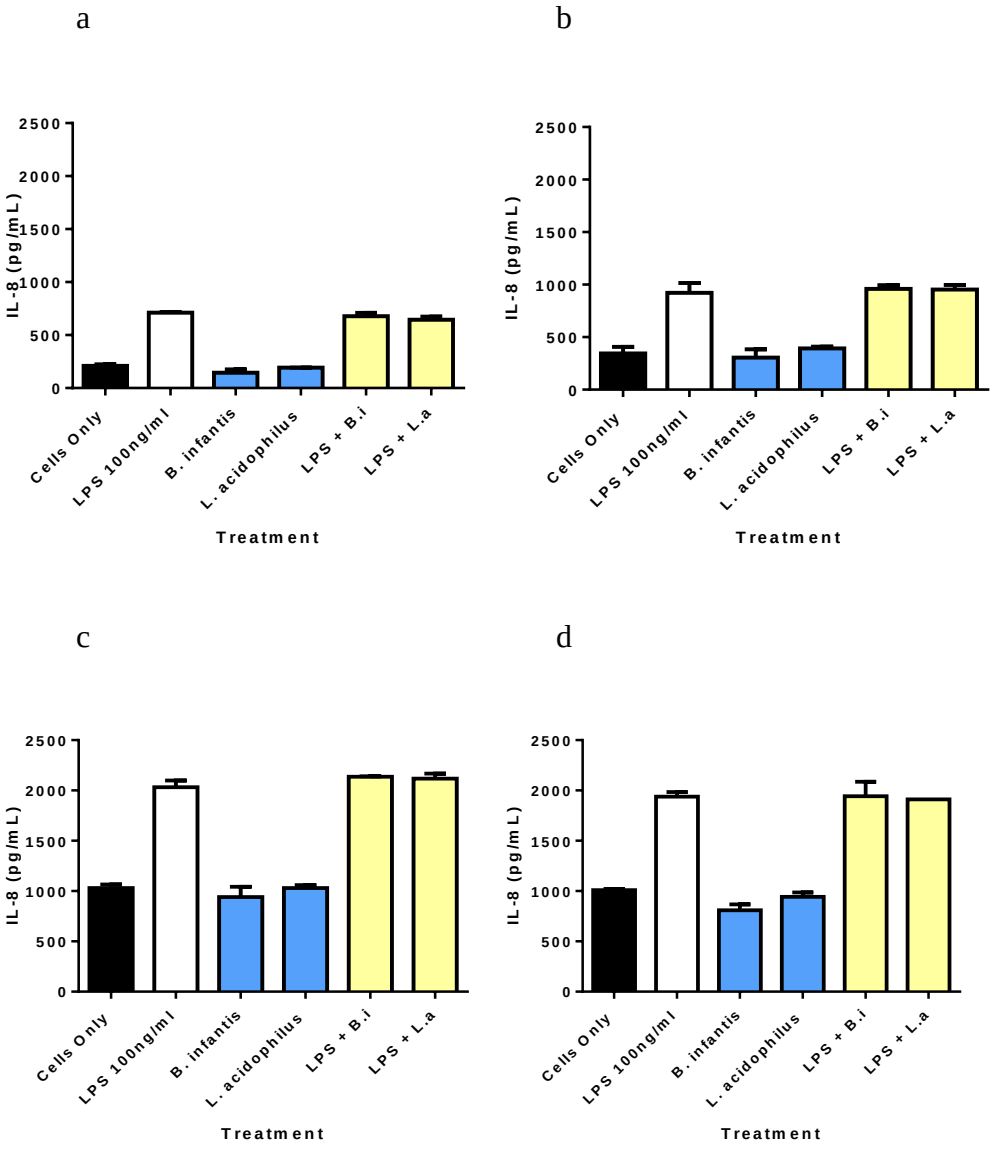


Figure 5.3. Heat-killed probiotics and the IL-8 production by HT-29 cells. Semi-confluent HT-29 cells were incubated for 6 (a), 8 (b), 20 (c) and 24 h (d) with $1x10^7$ cfu/mL of probiotic bacteria and 100 ng/mL LPS. Media only and LPS only treated cells were used as the negative and positive controls respectively. Bars and error bars represent the mean and the SEM of duplicate wells of one experiment. + B.i indicates a co-incubation with *B. infantis* and + L.a indicates a co-incubation with *L. acidophilus*.

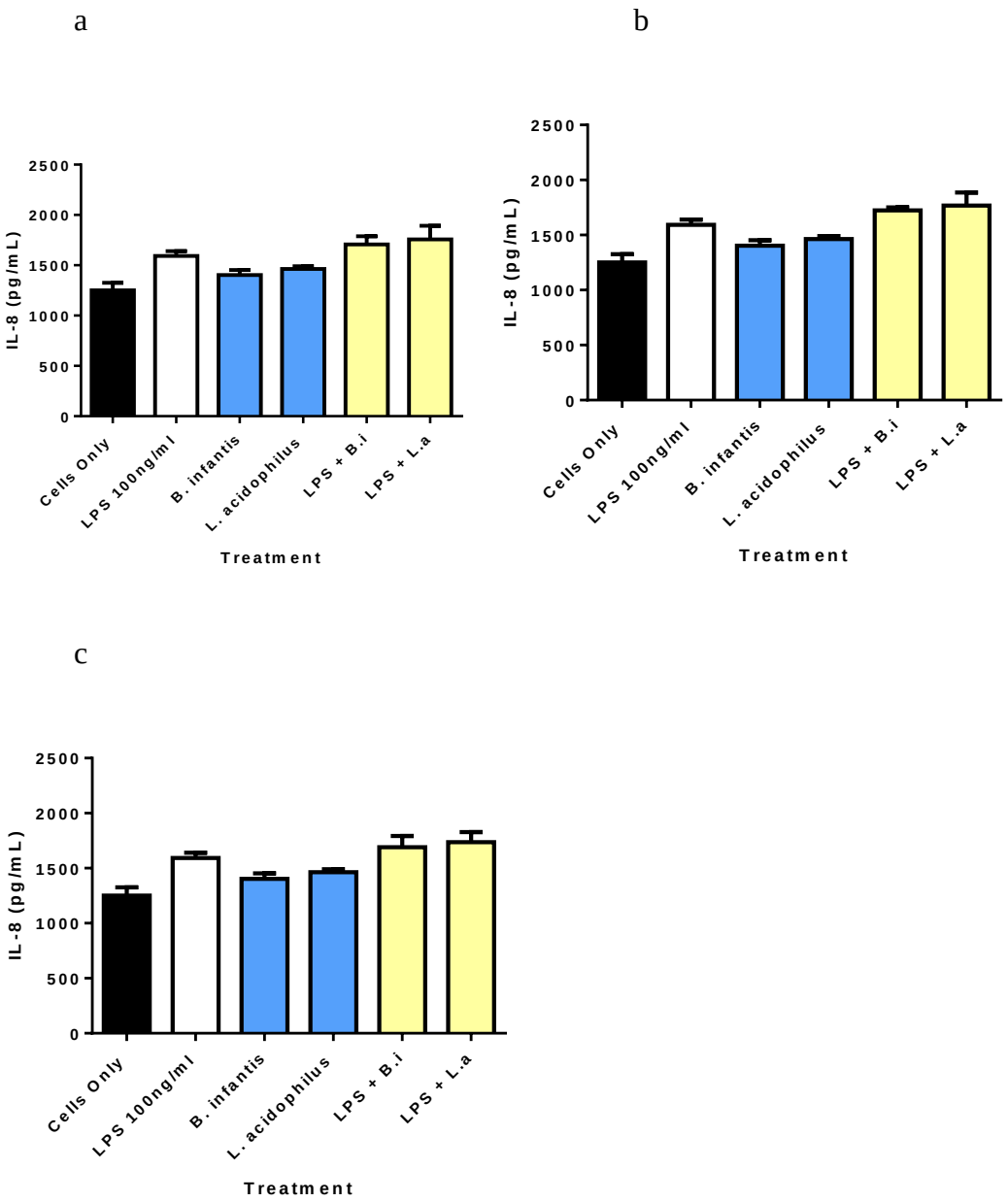


Figure 5.4. Probiotic prime and IL-8 production by HT-29 cells. Semi-confluent HT-29 cells were incubated for 6 (a), 3 (b) or 1 h (c) with $1x10^7$ cfu/mL of probiotic bacteria prior to the 24 h incubation with 100 ng/mL LPS. Media only and 100 ng/mL LPS only treated cells were used as the negative and positive controls respectively. Bars and error bars represent the pooled mean and the SEM of duplicate wells of two separate experiments. + B.i indicates a prime with *B. infantis* and + L.a indicates a prime with *L. acidophilus*.

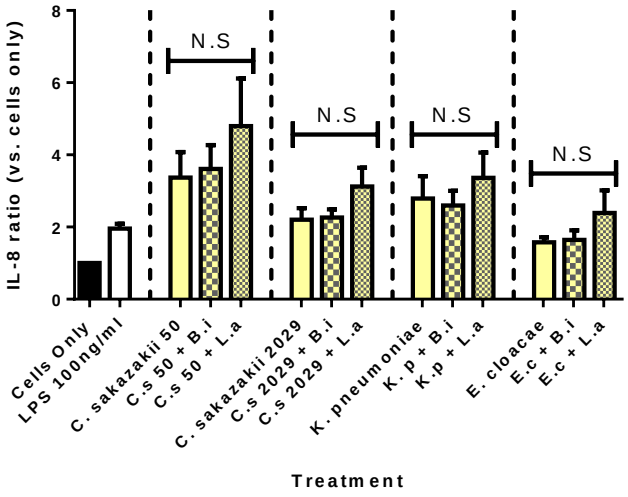


Figure 5.5. Heat-killed probiotic bacteria and the IL-8 production by HT-29 cells in response to NEC-associated bacteria. Semi-confluent HT-29 cells were incubated for 24 h with $1x10^7$ cfu/mL of probiotic bacteria prior to the addition and 24 h incubation with $1x10^7$ cfu/mL heat-killed NEC-associated bacteria. Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Statistical analysis; one way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S = No significant difference between treatments within this group. Bars and error bars represent the pooled mean and SEM of duplicate wells of three separate experiments. + B.i indicates a prime with *B. infantis* and + L.a indicates a prime with *L. acidophilus*.

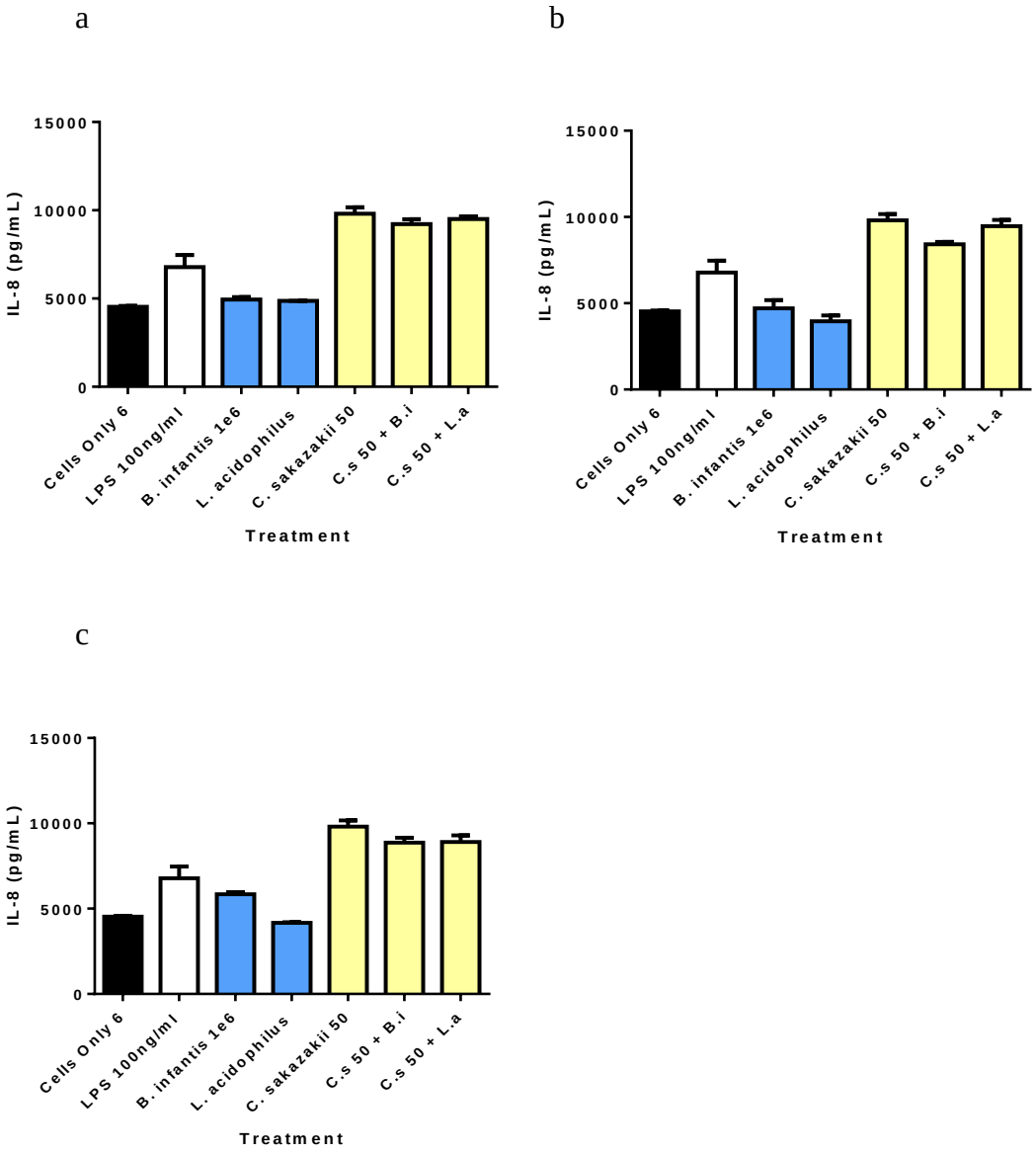
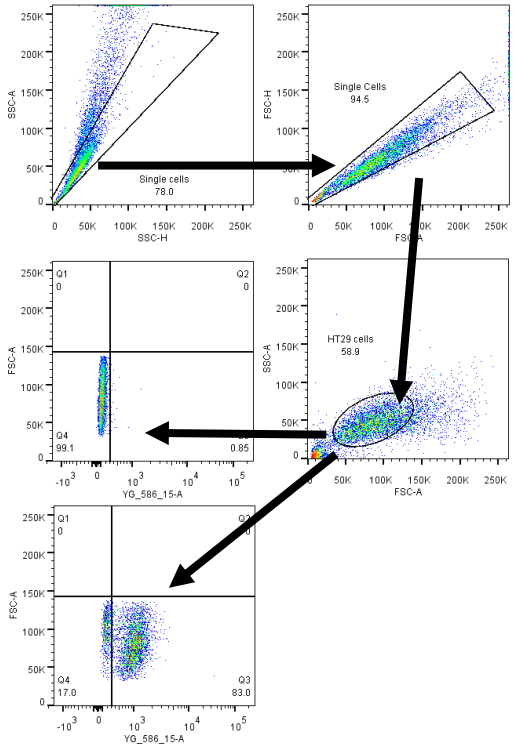
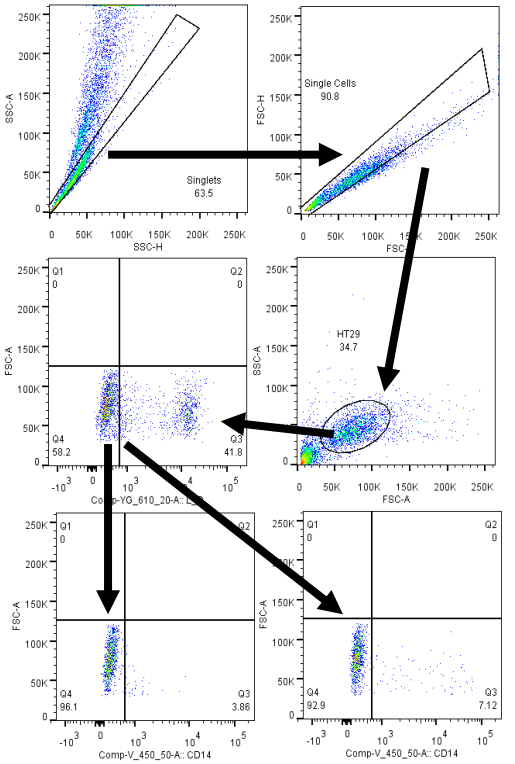


Figure 5.6. Different concentrations of heat-killed probiotic bacteria and the IL-8 production by HT-29 cells in response to *Cronobacter sakazakii* 50. Semi-confluent HT-29 cells were incubated for 6 h with $1x10^6$ (a) $1x10^7$ (b) or $1x10^8$ (c) cfu/mL of probiotic bacteria prior to the addition and 24 h incubation with $1x10^7$ cfu/mL heat-killed *C. sakazakii* 50. Media only and 100 ng/mL LPS were used as the negative and positive controls respectively. Bars and error bars represent the mean and the SEM of duplicate wells of one experiment. + B.i indicates a co-incubation with *B. infantis* and + L.a indicates a co-incubation with *L. acidophilus*.

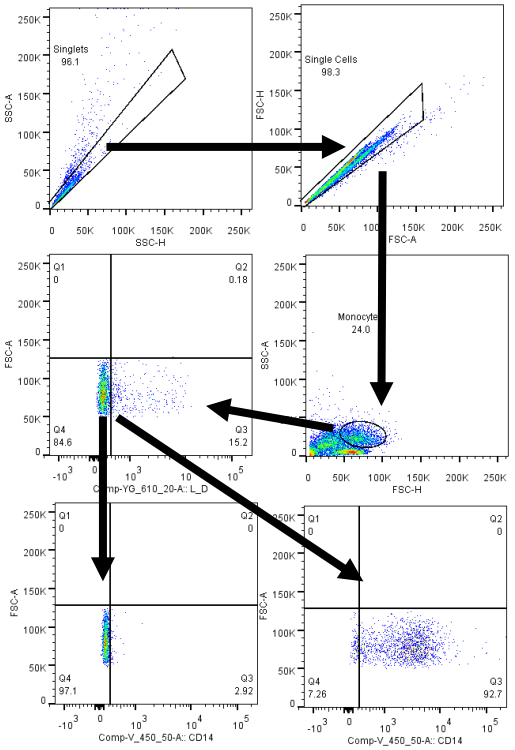
a


Single cells
78.0
Single Cells
94.5
HT29 cells
58.9
Q4
99.1
0.85
Q4
17.0
Q3
83.0
YG_586_15-A
FSC-A
SSC-A
FSC-H
SSC-H

b


Singlets
63.5
Single Cells
90.8
HT29
34.7
Q4
58.2
Q3
41.8
Q4
96.1
Q3
3.86
Q4
92.9
Q3
7.12
Comp-V_450_50-A:: CD14
FSC-A
SSC-A
FSC-H
SSC-H

c


Singlets
96.1
Single Cells
98.3
Monocyte
24.0
Q4
84.6
Q3
15.2
Q4
97.1
Q3
2.92
Q4
7.26
Q3
92.7
Comp-V_450_50-A:: CD14
FSC-A
SSC-A
FSC-H
SSC-H

d

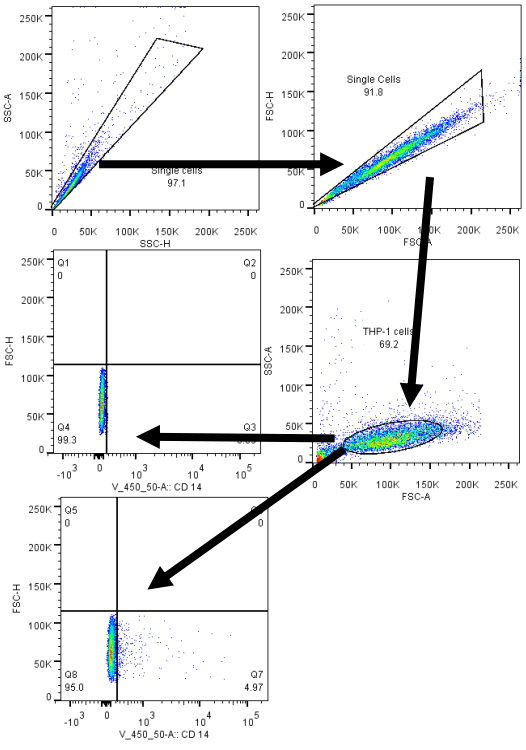

Single cells
97.1
Single Cells
91.8
THP-1 cells
69.2
Q4
99.3
Q8
95.0
Q7
4.97
V_450_50-A:: CD14
FSC-A
SSC-A
FSC-H
SSC-H

Figure 5.7. Gating strategies for various cell types for flow cytometry analysis. Gating strategies used in FlowJo software for flow cytometry analysis are depicted for HT-29 cells, HT-29 cells with a live dead and brilliant violet stain, PBMCs and THP-1 cells. HT-29, PBMC and THP-1 cells were stained with an anti-CD14 (PE or brilliant violet) or isotype control antibody or left unstained and analysed by flow cytometry. Flow cytometric data was analysed in the software FlowJo. Numbers beside gates indicate the percentage of total cells within that gate. Quad gates were gated on unstained BV or PE fluorescent negative populations. Ten thousand events were recorded for each treatment during each experiment.

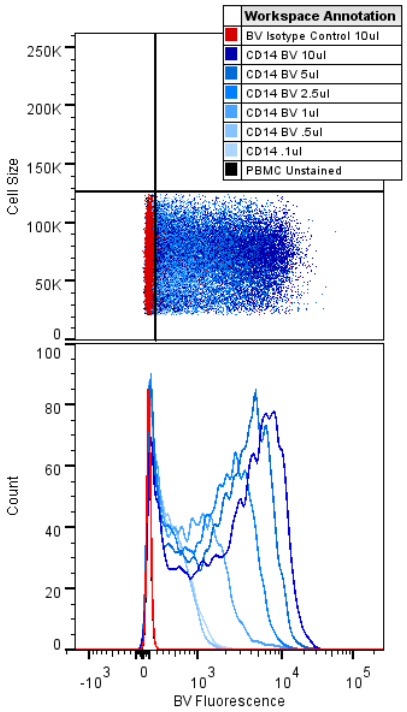


Figure 5.8. Brilliant violet antibody titration. Antibody titrations are a critical step in ensuring flow cytometric analysis is correct. Titrations ensure the optimal amount of antibody is used in experiments. The brilliant violet anti-CD14 antibody and isotype was titrated on the PBMC CD14 positive control. PBMCs were stained with 10 to 0.1 µL of brilliant violet anti-CD14 or isotype control antibody in 100 µL of diluent or left unstained and analysed by flow cytometry. Flow cytometric data was analysed on the software FlowJo. Ten thousand events were recorded for each treatment.

M +ve 0 1 10 100 1000

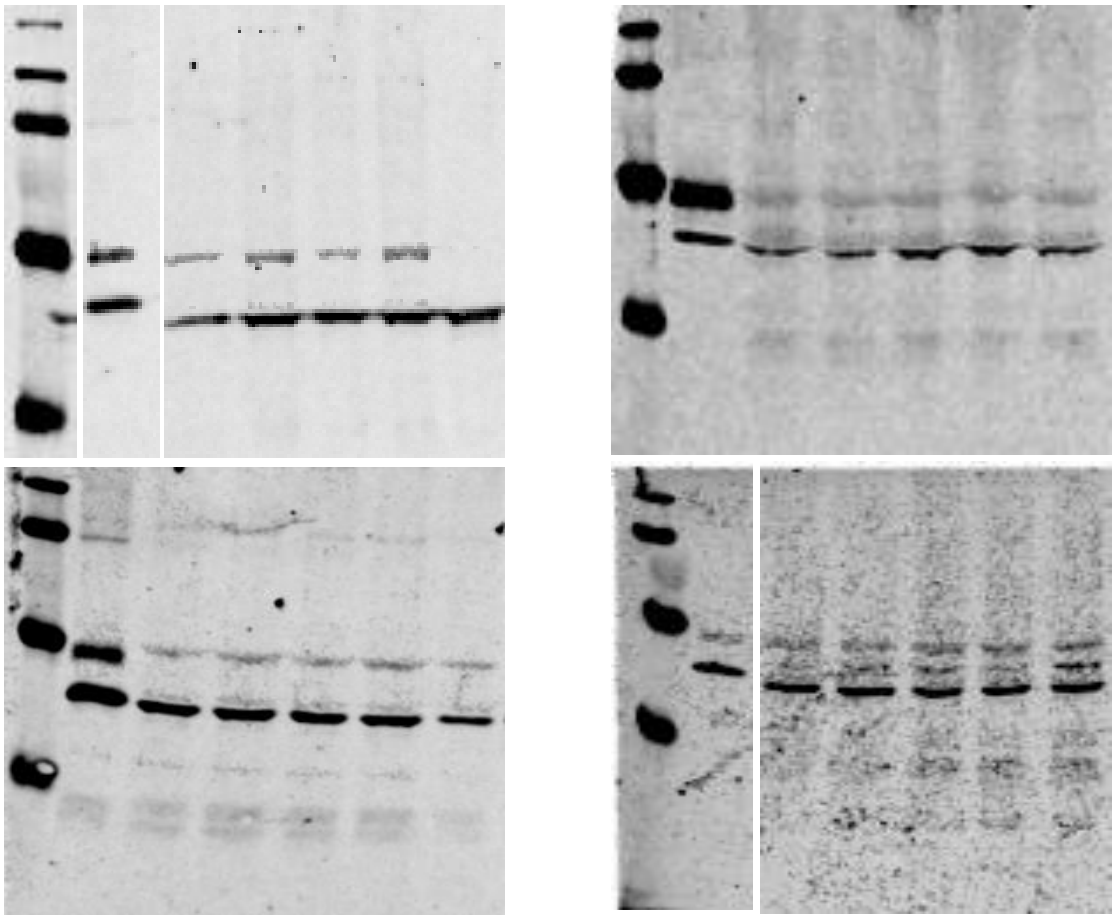


Figure 5.9. CD14 expression by HT-29 cells. Western blot images showing the presence of CD14. Lanes from left to right in each blot shows molecular weight marker (M), PBMC CD14 positive control (+ve), 0, 1, 10, 100 and 1000 ng/mL LPS respectively HT-29 cells were stimulated with during experiment. Actin served as a loading control.

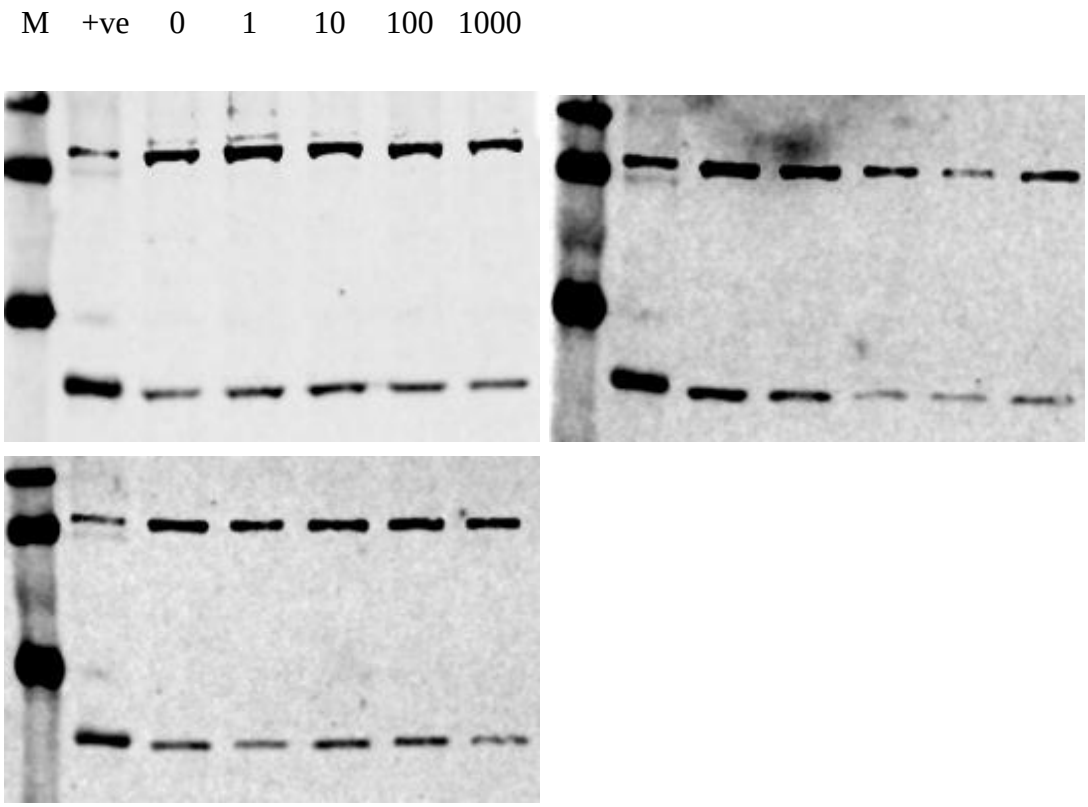


Figure 5.10. TLR4 expression by HT-29 cells. Western blot image showing the presence of TLR4. Lanes from left to right represent the molecular weight marker (M), PBMC TLR4 positive control (+ve), 0, 1, 10, 100 and 1000 ng/mL LPS respectively HT-29 cells were stimulated with during assay. Actin served as a loading control. Molecular weight marker indicates approximately 130 kDa (highest band), 100 kDa (middle band) and 55 kDa (lowest band).

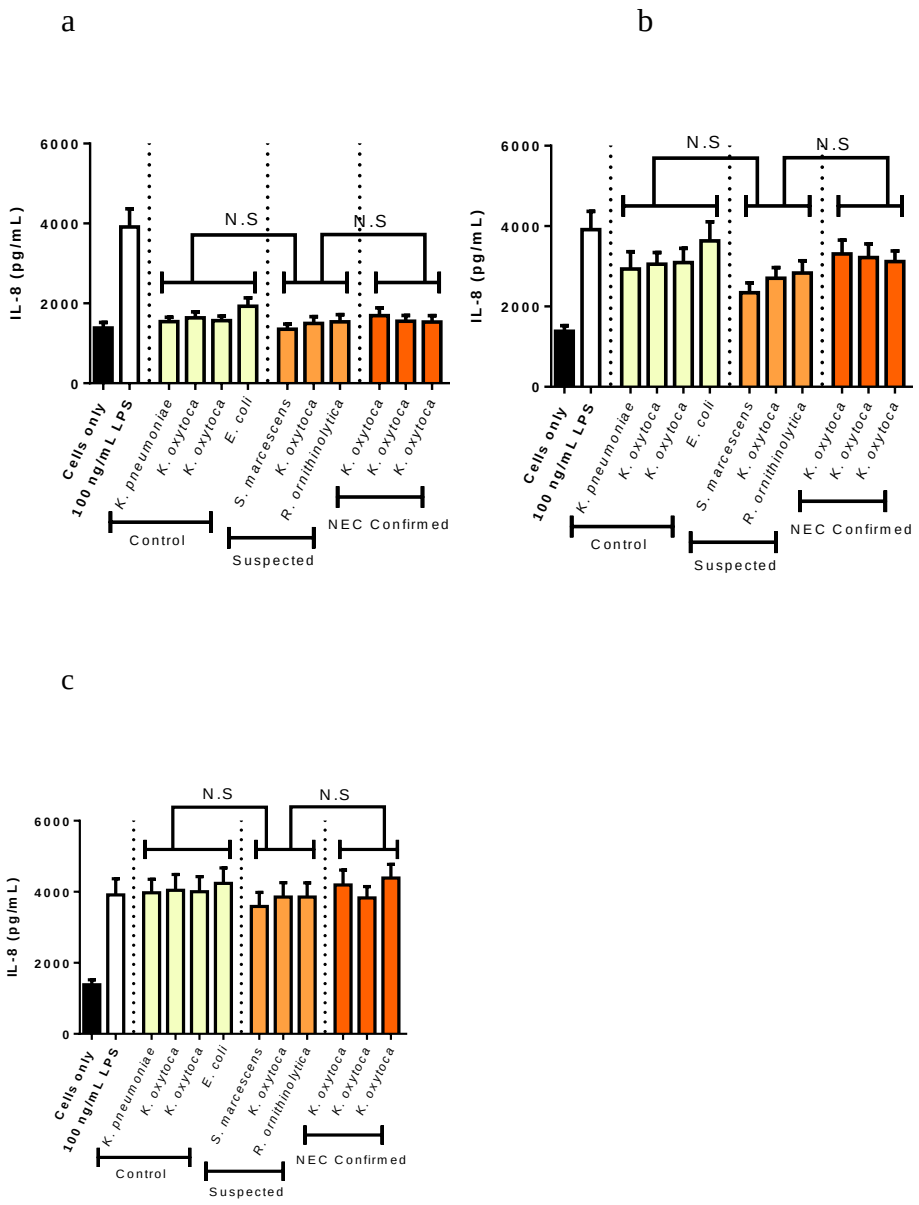


Figure 5.11. IL-8 production by HT-29 cells in response to clinically isolated bacteria from different NEC group infants. Semi-confluent HT-29 cells were incubated with $1x10^6$ (a) $1x10^7$ (b) or $1x10^8$ cfu/mL (c) of 10 different strains of washed, heat-killed *Enterobacteriaceae* for 24 h. Bacteria were originally isolated from control, suspected or NEC confirmed infants. Media only and 100 ng/mL LPS was used as the negative and positive controls respectively. Statistical analysis; one way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S, no significant difference between any treatments within this group compared to any treatments within the indicated group. Bars and error bars represent the pooled mean and the SEM of duplicate wells of four separate experiments.

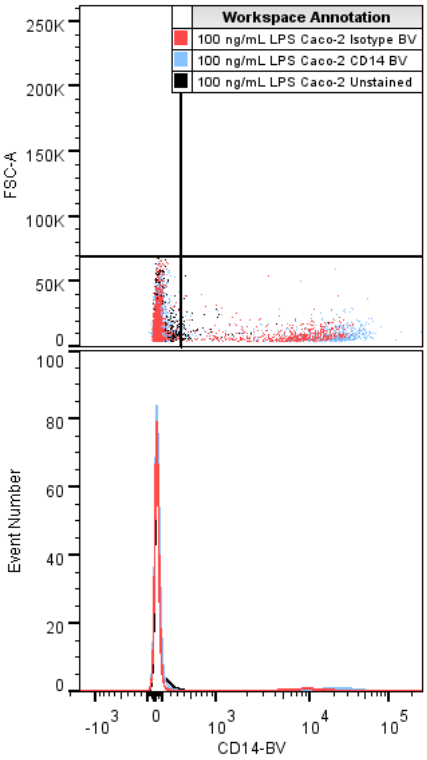


Figure 5.12. CD14 expression on stimulated Caco-2 cells by flow cytometry. Semi-confluent Caco-2 cells were cultured with 100 ng/mL LPS for 24 hours and stained with an anti-CD14 or isotype control antibody or left unstained. Ten thousand events were recorded.

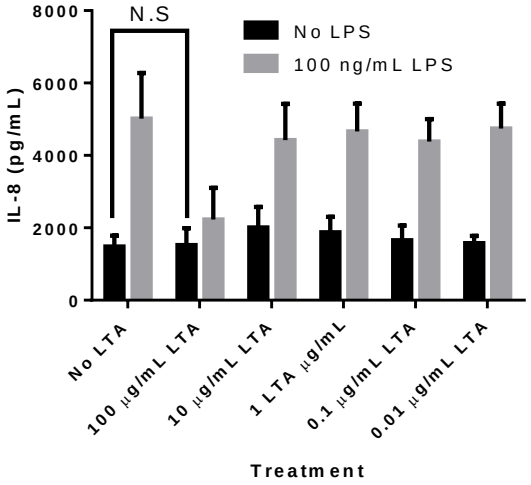


Figure 5.13. LTA and unstimulated HT-29 cells. Semi-confluent HT-29 cells were incubated for 24 h with various concentrations of LTA with or without 100 ng/mL of *E. coli* LPS. Statistical analysis; ordinary two way ANOVA with multiple comparisons (Tukey's multiple comparisons test). N.S, no significant difference between the two treatments. Bars and error bars represent the pooled mean and SEM of duplicate wells of three separate experiments.

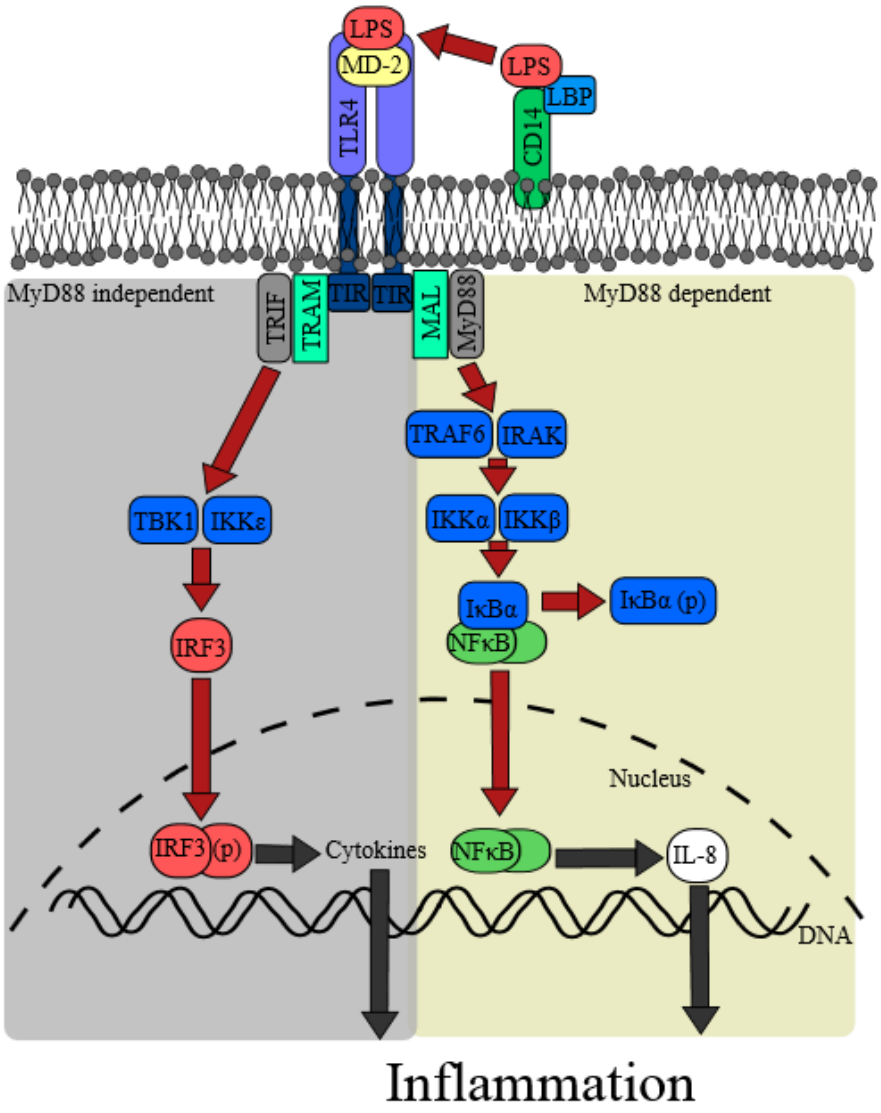


Figure 5.14 Simplified diagram of LPS induced intracellular signalling in intestinal epithelial cells. During the recognition of LPS by immune cells or IECs, LBP recruits LPS to CD14. It is then passed to a TLR4 dimer that is associated with the adapter molecule MD-2. Intracellular signalling pathways activated are MyD88 dependent or independent. MyD88 dependent signalling requires the involvement of TRAF6, IRAK molecules and IκB kinases. When IκBα is phosphorylated, it no longer blocks the transcription factor, NF-κB. This allows NF-κB to translocate into the nucleus and induce pro-inflammatory gene production, which includes IL-8. Alternatively, through the action of TRIF, TRAM, TBK and IKKε, IRF becomes phosphorylated and dimerizes, allowing it to translocate into the nucleus and induce cytokine gene transcription. Production of pro-inflammatory molecules by IECs contributes to the pro-inflammatory response seen during NEC. Abbreviations: LPS, lipopolysaccharide;

LBP, lipopolysaccharide binding protein; CD14, cluster of differentiation 14; MD-2, myeloid differentiation protein 2; TLR4, toll like receptor 4; TIR, Toll-Interleukin receptor domain; MyD88, myeloid differentiation marker; MAL, MyD88 adapter like protein; TRIF, TIR containing adapter; TRAM, TRIF like adapter molecule, TRAF, tumour necrosis factor receptor associated factor; IRAK, Interleukin-1 receptor-associated kinase; IKK, IκB kinase; IκBα, inhibitor of kappa B protein; NF-κB, nuclear factor κB; TBK, TANK binding kinase; IRF, Interferon regulatory factor; DNA, deoxyribonucleic acid. (p) indicates phosphorylation. Adapted from Palsson-McDermott and O'Neill[111] and O'Neill et al.[226]

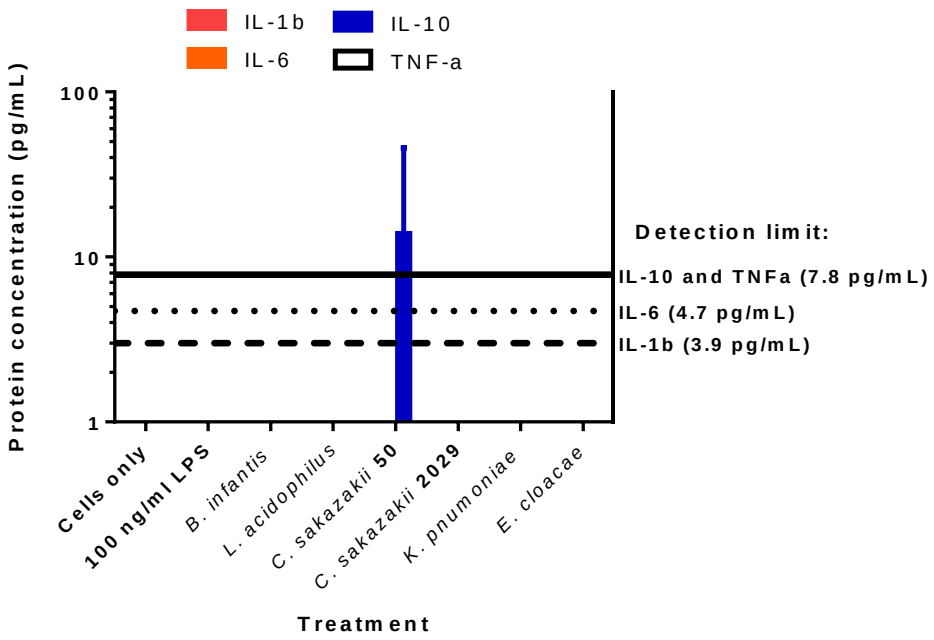


Figure 5.15. Production of IL-1β, IL-6, IL-10 and TNF-α by HT-29 cells. Semi-confluent HT-29 cells were incubated for 24 with 100 ng/mL LPS, $1x10^7$ cfu/mL of heat inactivated Gram negative NEC bacteria or $1x10^7$ cfu/mL heat inactivated probiotic bacteria. Media only was used as the negative control. Bars and error bars represent the mean and the SEM of duplicate wells of one experiment. Detection limits of each ELISA assay is depicted.

Table 5.1 Different techniques undertaken in order to effectively measure CD14 expression on HT-29 cells without non-specific binding

Technique used:	Details:	Effective? Yes/No
Range of isotype control antibodies	PE mouse IgG2a-κ	No
	PE rat IgG2a-κ	No
	PE mouse IgG-κ	No
FcR block	Human TruStain FcX	No
Antibody titrations	0, 0.1, 0.5, 1, 2.5, 5 or 10 µL per 100 µL	No
Live/dead stain	Live/Dead® Fixable Red Dead Cell Stain	No
Different dissociation methods	Cell scraper, BD Falcon™	No
	Trypsin: Gibco®, Life Technologies™	No
	Enzyme free dissociation buffer: Gibco®, Life Technologies	No
	TrypLE: Gibco®, Life Technologies™	No
Intracellular staining		No
Change staining and isotype control antibody	Brilliant Violet mouse anti-human CD14 (Clone: M5E2, BioLegend) and Brilliant Violet IgG-κ isotype control (Clone: MOPC-173, BioLegend)	Yes

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